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Review

Liver fibrosis in NAFLD/NASH: from pathophysiology towards diagnostic and therapeutic strategies

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ABSTRACT

Liver fibrosis, as an excess deposition of extracellular matrix (ECM) components, results from chronic liver injury as well as persistent activation of inflammatory response and of fibrogenesis. Liver fibrosis is a major determinant for chronic liver disease (CLD) progression and in the last two decades our understanding on the major molecular and cellular mechanisms underlying the fibrogenic progression of CLD has dramatically improved, boosting pre-clinical studies and clinical trials designed to find novel therapeutic approaches. From these studies several critical concepts have emerged, starting to reveal the complexity of the pro-fibrotic microenvironment which involves very complex, dynamic and interrelated interactions between different hepatic and extrahepatic cell populations. This review will offer first a recapitulation of established and novel pathophysiological basic principles and concepts by intentionally focus the attention on NAFLD/NASH, a metabolic-related form of CLD with a high impact on the general population and emerging as a leading cause of CLD worldwide. NAFLD/NASH-related pro-inflammatory and profibrogenic mechanisms will be analysed as well as novel information on cells, mediators and signalling pathways which have taken advantage from novel methodological approaches and techniques (single cell genomics, imaging mass cytometry, novel in vitro two- and three-dimensional models, etc.). We will next offer an overview on recent advancement in diagnostic and prognostic tools, including serum biomarkers and polygenic scores, to support the analysis of liver biopsies. Finally, this review will provide an analysis of current and emerging therapies for the treatment of NAFLD/NASH patients.

1. Introductory remarks on liver fibrosis and fibrogenesis

Liver fibrosis, as detected in progressive chronic liver diseases (CLD), can be defined as an excess deposition of extracellular matrix (ECM) components associated with parallel matrix degradation and remodelling. It represents a common pathological outcome of different etiological conditions resulting in chronic parenchymal injury and persistent and sustained activation of inflammatory response as well as chronic wound healing and liver fibrogenesis (Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Koyama and Brenner, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Friedman and Pinzani, 2022).

According to epidemiological studies, liver fibrosis can develop mainly as a consequence of three major conditions that result in chronic liver injury (Byass, 2014; Marcellin and Kutala, 2018; Thrift et al., 2017; Younossi et al., 2018; Golabi et al., 2023), including: i) chronic infection by hepatotropic viruses (mainly hepatitis B and C viruses, or HBV and HCV); ii) chronic toxic injury, mainly due to excess alcohol consumption; iii) metabolic-related injury, with non-alcoholic fatty liver disease (NAFLD) and its progressive form of non-alcoholic steatohepatitis (NASH). NAFLD/NASH is rapidly emerging as the worldwide leading cause of CLD for the next decades (Younossi et al., 2018; Golabi et al., 2023), also as a consequence of the remarkable success of direct antiviral agents (DAA) in treating chronic viral hepatitis, particularly HCV infection (Falade-Nwulia et al., 2017; EASL, 2018a; AASLD/IDSA, 2018; Rockey and Friedman, 2021). Other less common aetiologies include hepatic autoimmune diseases involving, as primary cellular targets, either hepatocytes in autoimmune hepatitis (AIH) or cholangiocytes in primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) (Arndtz and Hirschfield, 2016) as well as a limited number of chronic cholangiopathies (Lazaridis and LaRusso, 2015; Cannito et al., 2018) and hereditary diseases (mainly hereditary hemochromatosis, Wilson's disease and α1-antitrypsin deficiency) (Byass, 2014; Marcellin and Kutala, 2018).

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Irrespective of the specific aetiology, liver fibrosis is currently believed to offer a major contribution to CLD progression towards the advanced stage of clinically evident cirrhosis with portal hypertension and related complications (formation of vascular shunts and variceal bleeding, development of ascites, hepatic encephalopathy and hepatorenal syndrome), eventually leading to hepatic failure (Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Koyama and Brenner, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Friedman and Pinzani, 2022). In addition, CLD progression towards cirrhosis is also related to an increased risk to develop hepatocellular carcinoma (HCC), the most common primary liver cancer (i.e., the sixth most common cancer and the fourth leading cause of cancer mortality worldwide). Accordingly, more than 90% of HCC cases occur in the setting of a progressive CLD, with liver cirrhosis, irrespective of aetiology, being the major risk factor for HCC (EASL, 2018b; Marrero et al., 2018a; Llovet et al., 2021; Llovet et al., 2023). However, approx. 25–30% of NASH-associated HCC can occur in the absence of a diagnosis of cirrhosis as in NASH patients with advanced fibrosis (Llovet et al., 2023).

The knowledge on the pathogenesis of liver fibrosis and its contribution to CLD progression has been remarkably expanded in the last decades by a very impressive number of basic experimental, translational and clinical studies. In particular, several critical concepts have emerged from more recent studies dedicated to the metabolic-related condition of NAFLD/NASH, starting to reveal the complexity of the so-called *pro-fibrotic microenvironment* which involves very complex, dynamic and interrelated interactions (i.e., in terms of molecular mediators and signalling pathways) between different hepatic and extrahepatic cell populations. Along these lines and as detailed in authoritative reviews (Trautwein et al., 2015; Higashi et al., 2017; Cannito et al., 2017a; Tacke, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Carter and Friedman, 2022; Peiseler et al., 2022; Wallace et al., 2022) activated hepatic myofibroblasts (MFs) and cells of either innate and adaptive immunity are known to play a major interconnected role in driving CLD fibrogenic progression. These recent remarkable achievements have also boosted additional pre-clinical studies and clinical trials designed to find novel therapeutic strategies that, at present, are mostly directed against NAFLD/NASH (Tacke, 2017; Asgharpour et al., 2021; Huisman et al., 2021; Friedman and Pinzani, 2022; Wiering and Tacke, 2022; Tacke et al., 2023).

However, despite this increased knowledge on the pathogenesis of liver fibrosis, we still lack approved antifibrotic therapies for CLD and, as recently reviewed by Friedman and Pinzani (2022), there are still several unmet needs that basic, translational and clinical studies should clarify in the future. In his review we will first offer a synthetic recapitulation of basic principles and concepts by intentionally focus the attention on NAFLD/NASH, a metabolic-related form of CLD with a high impact on the general population and considered as the emerging leading cause of CLD worldwide for the next decades. Accordingly, we will analyse established and emerging pro-inflammatory and profibrogenic mechanisms as well as novel information on cells, mediators, and signalling pathways in the fibrogenic progression of NASH that have taken advantage from the availability of advanced methodological approaches and techniques (single cell genomics, imaging mass cytometry, novel in vitro two- and three-dimensional models, etc.). We will next offer an overview on recent advancement in diagnostic and prognostic tools to finally provide an analysis of current and emerging therapies for the treatment of NAFLD/NASH patients.

2. NAFLD: the emerging leading cause of CLD

2.1. Epidemiology of NAFLD

In the last decade several studies have outlined and supported the notion that NAFLD represents the emerging leading cause of CLD worldwide (Loomba and Sanyal, 2013; Younossi et al., 2016; Younossi et al., 2018; Younossi et al. 2019a; Golabi et al., 2023). From epidemiological studies NAFLD was believed to affect approx. 1 billion of individuals worldwide, with a global prevalence of approx. 25% in the general population, with geographic variations ranging from 13% in Africa up to 42% in southeast Asia (Younossi et al., 2018; Younossi et al. 2019a; Golabi et al., 2023). However, a more recent and accurate meta-analysis, covering the 1990–2019 period and performed by pooling NAFLD prevalence estimates and ultrasound-defined NAFLD, revealed an even higher overall global prevalence of 30.05% (95% CI: 27.88%–32.32%) and 30.69% (28.4–33.09), respectively (Younossi et al., 2023). It has been estimated that just in the United States NAFLD may affect as much as 80 million of individuals; in particular, in USA, Europe and Southeast Asia, the NAFLD prevalence is estimated to further raise by 2030 by affecting in these areas more than 400 million of individuals (Estes et al., 2018). Moreover, the prevalence and incidence of NAFLD has been reported to rapidly increase in adults, children and adolescents in Europe, USA, China, India and other regions, making NAFLD a global health problem.

The high global prevalence of NAFLD has been proposed to parallel the worldwide rising rates of obesity and type 2 diabetes (T2D), with NAFLD being currently viewed as the hepatic manifestation of the metabolic syndrome and then associated with additional metabolic risk factors (Marchesini et al., 2001; Neuschwander-Tetri et al., 2010; Chalasani et al., 2018; Samuel and Shulman, 2018; Chakravarthy and Neuschwander-Tetri, 2020). However, it should be noted that not all obese individuals develop NAFLD and that NAFLD can be also detected in non-obese adults and children of all ethnicities, with the reported prevalence ranging from 3% to 30% but with a higher prevalence found particularly in non-obese male Asian individuals (Kim and Kim, 2017). Along these lines, it has been reported that in USA 43% of individuals with NAFLD are not obese, a feature rising to 71% in Sweden (Ye et al., 2020). Moreover, histopathological studies on liver biopsies suggest that the prevalence of NASH and fibrosis does not significantly differ between NAFLD in obese individuals and non-obese NAFLD patients (Kim and Kim, 2017).

2.2. NAFLD: definition, diagnosis, and natural history

NAFLD can be diagnosed when histopathological analysis of liver biopsies or imaging techniques indicate the presence of more than 5% of hepatocytes with accumulation of fat in patients who consume little or no alcohol and when one can exclude the involvement other metabolic, toxic- or drug-induced causes of fatty liver (Chalasani et al., 2018). From a histopathological point of view, NAFLD can be defined as a spectrum of conditions ranging from simple steatosis or fatty liver to the progressive form of non-alcoholic steatohepatitis or NASH. Simple steatosis, sometimes referred to as non-alcoholic fatty liver (NAFL) and detected in approx. 75–80% of NAFLD patients, is benign and usually viewed as a not progressive or slowly progressive condition (Singh et al., 2015; Chalasani et al., 2018). In turn, the histopathological diagnosis of NASH requires the evidence of liver steatosis associated with aspects of parenchymal injury such as ballooning, apoptosis (or lipo-apoptosis), focal necrosis, lobular and/or portal inflammatory infiltrate and a variable degree of fibrosis. NASH affects the remaining NAFLD patients and NASH patients are at high risk to undergo fibrogenic progression towards a more advanced stage of CLD, with approx. 15–20% of NASH patients developing cirrhosis usually over 3-4 decades (Singh et al., 2015; Satapathy and Sanyal, 2015; McPherson et al., 2015; Younossi et al., 2019a; Loomba and Adams, 2019; Powell et al., 2021). Of interest, NASH-associated liver fibrosis has been identified as the strongest predictor for disease-specific progression and mortality (Powell et al., 2021). It should be noted that cardiovascular disease is by far the leading cause of mortality in NAFLD patients, followed by cancer and then liver failure due to CLD progression (Adams et al., 2005). In obese NASH patients the risk for cardiovascular disease can be even 10-fold higher than in non-obese ones (Yoshitaka et al., 2017) but CLD

becomes the primary cause of mortality for NASH with a diagnosis of cirrhosis.

In addition, cirrhotic NASH patients carry a 1.3–2% per year risk of developing HCC (Loomba et al., 2020) and overall NASH is now indicated as the most rapidly growing cause of HCC in liver transplant candidates (Younossi et al., 2019b; Huang et al., 2022). The latter issue is of relevance: although the incidence of NASH-related HCC is still lower than that of HCC of other aetiologies, the effectiveness of therapies for chronic hepatic viral infections is significantly changing the global scenario. The incidence of NASH-related HCC is increasing significantly worldwide and estimated to represent the prevalent cause of HCC in the next decades (Negro, 2020; Huang et al., 2021, 2022). Moreover, as previously mentioned, about 25–30% of NASH-associated HCC have been reported to occur in non-cirrhotic patients, which is a rate much higher than for other aetiologies of CLD (Llovet et al., 2023). The latter consideration represents an additional burden for a disease that has such a relevant impact on the general population: one should consider that the majority of individuals with NAFLD are usually asymptomatic and then the disease may remain silent until the progression to cirrhosis has occurred (Spengler and Loomba, 2015; Chalasani et al., 2018; Loomba and Adams, 2019). Moreover, although current diagnostic tools are improving, we still lack reliable biomarkers able to specifically evaluate and predict the individual risk of progression (i.e., towards cirrhosis and/or HCC development) for a NASH patient (Friedman and Pinzani, 2022). In this context, it should be noted that there are no specific surveillance guidelines for the detection of early HCC in non-cirrhotic NASH patients at present. Finally, although an impressive number of dedicated clinical trials has been performed in the last decade and several others are currently ongoing, no selective therapy for NAFLD/NASH has been validated until now and, pertinent to this review, there are no drugs approved to specifically treat hepatic fibrosis in NASH or any other CLD (Huisman et al., 2021; Dufour et al., 2022; Friedman and Pinzani, 2022; Wiering and Tacke, 2022; Ratziu et al., 2022; Harrison et al., 2023a).

2.3. The new nomenclature of NAFLD

To make more clarity in the definition of NAFLD thus avoiding the stigmatizing terms “non-alcoholic” and “fatty” and facilitating a more homogeneous recruitment in clinical trials, a multi-society Delphi consensus statement was released in June 2023 during the annual meeting of the European Association for the Study of the Liver (EASL) (Rinella et al., 2023). The term “fatty liver” has been changed with “steatotic liver disease” (SLD). What was in most cases generically indicated for NAFLD has been replaced with Metabolic Dysfunction Associated Steatotic Liver Disease (MASLD) in the presence of at least one cardiometabolic risk factor (i.e., high values or levels of one between: body mass index or BMI, fasting serum glucose, blood pressure, plasma triglycerides, plasma HDL-cholesterol) in addition to hepatic steatosis and in the absence of significant alcohol intake (140–350 g/week and 210–420 g/week for females and males, respectively). Accordingly, the presence of cardiometabolic risk factors associated with a significant and increasing alcohol intake now configures a new entity termed Metabolic Alcohol Associated Liver Disease (MetALD). The consensus statement also allocates more precisely other manifestations of steatosis which are independent of metabolic risk factors. The term steatohepatitis was felt to be an important pathophysiological concept that should be retained. Metabolic dysfunction-Associated SteatoHepatitis (MASH) was then proposed to replace the old term non-alcoholic steatohepatitis (NASH). While it was important to highlight this very recent key advancement, the term NAFLD/NASH will be consistently used in this article meaning to a very large extent what is now defined as MASLD/MASH. Accordigly, application of the new nomenclature allows the preservation of existing data on natural history, biomarkers and clinical trials considering that 98% of the existing Registry cohorts of patients with NAFLD would fulfill the new criteria for MASLD.

3. NAFLD as a disease resulting from dysregulated metabolism, lipotoxicity, genetic variants and changes in gut microbiome

3.1. Metabolic causes of NAFLD

As previously mentioned, NAFLD is closely linked to obesity and type 2 diabetes and, as nicely described in an excellent review by Loomba et al. (2021), the metabolic mechanisms resulting in NAFLD reflect an imbalance of liver energy metabolism due to an excess delivery of lipids and carbohydrates to the organ that overcomes its ability to oxidize these substrates or to export them as incorporated into very low-density lipoproteins (VLDL). Overall, TG accumulation in hepatocytes may be seen then as a form of surplus energy accumulation in the liver also because of the deficiency of lipid storage in white adipose tissue (WAT) (Loomba et al., 2021).

Along these lines, we will here just briefly recapitulate major issues related to the metabolic mechanisms leading to NAFLD. Interested readers can refer for more mechanistic details (mediators, pathways, molecular mechanisms, etc.) to authoritative reviews that will be indicated for any relevant issue. As synthetized in Fig. 1A, the excess delivery of lipids and carbohydrates to the liver is the results of the following major issues: i) an increased dietary intake of food containing high levels of lipids, particularly saturated fatty acids, and/or high levels of carbohydrates like mono- and di-saccharides, particularly fructose and sucrose; an excess intake of carbohydrates (typical in the so-called western diet), particularly of fructose which is mainly metabolized in the liver, can activate *de novo* lipogenesis that, in turn, can further exacerbate NAFLD (Herman and Samuel, 2016; Samuel and Shulman, 2018; Loomba et al., 2021); ii) the development of lipid deposition in skeletal muscle, that usually occurs prior to NAFLD onset, and the consequent development of local insulin resistance (IR); IR at the skeletal muscle then results in the inhibition of insulin signalling, decreased insulin-stimulated glucose transport and muscle glycogen synthesis, with dietary glucose, not stored as glycogen in the skeletal muscle, being redirected to the liver (Petersen et al., 2007; Flannery et al., 2012; Samuel and Shulman, 2018); iii) the development of hepatic IR which, in all forms of NASH, is strictly correlated with peripheral IR and is known to be aggravated during disease progression; with the time this results in an insufficient suppression of hepatic gluconeogenesis, a decreased glycogen synthesis (through impairment of glycogen synthase activation) and, by redirecting glucose into lipogenic pathways, leading to increased TG accumulation (Tilg et al., 2017; Samuel and Shulman, 2018); the association between IR and NAFLD, which is exacerbated in T2D patients, is an established issue and hepatic TG accumulation could originate either as a direct consequence of increased lipid delivery to the organ and/or from IR and hyperinsulinaemia (Marchesini et al., 2001; Roden, 2006; Gaggini et al., 2013; Tilg et al., 2017; Loomba et al., 2021); iv) the development of IR in the expanded and/or inflamed WAT, that results in increased lipolysis and increased fatty acid delivery to the liver which, in turn, can promote increased fatty acid esterification into TG and then NAFLD in a substrate-dependent but mostly insulin-independent manner. Progressive adipocyte dysfunction, apart from up-regulation of leptin expression, also involves genes linked to IR and dyslipidemia, such as genes linked to IR that affect the activity of lipoprotein lipase (Lpl) or genes that encode for components of insulin signalling pathway (Lotta et al., 2017). Additional features are believed to decrease peripheral TG clearance and increase hepatic uptake of TG from chylomicron remnants, like expression of endogenous Lpl inhibitors like ApoC3, lipoprotein-lipase inhibitor angiopoietin-related protein ANGPTL3/8 complex, and ANGPTL4 (Lotta et al., 2017; Samuel and Shulman, 2018; Loomba et al., 2021). One should note that also an increase in visceral adipose tissue (VAT), which occur in correlation with hyperlipidemia and IR, is believed to be causally related to NAFLD pathogenesis and related metabolic disturbances, leading to an

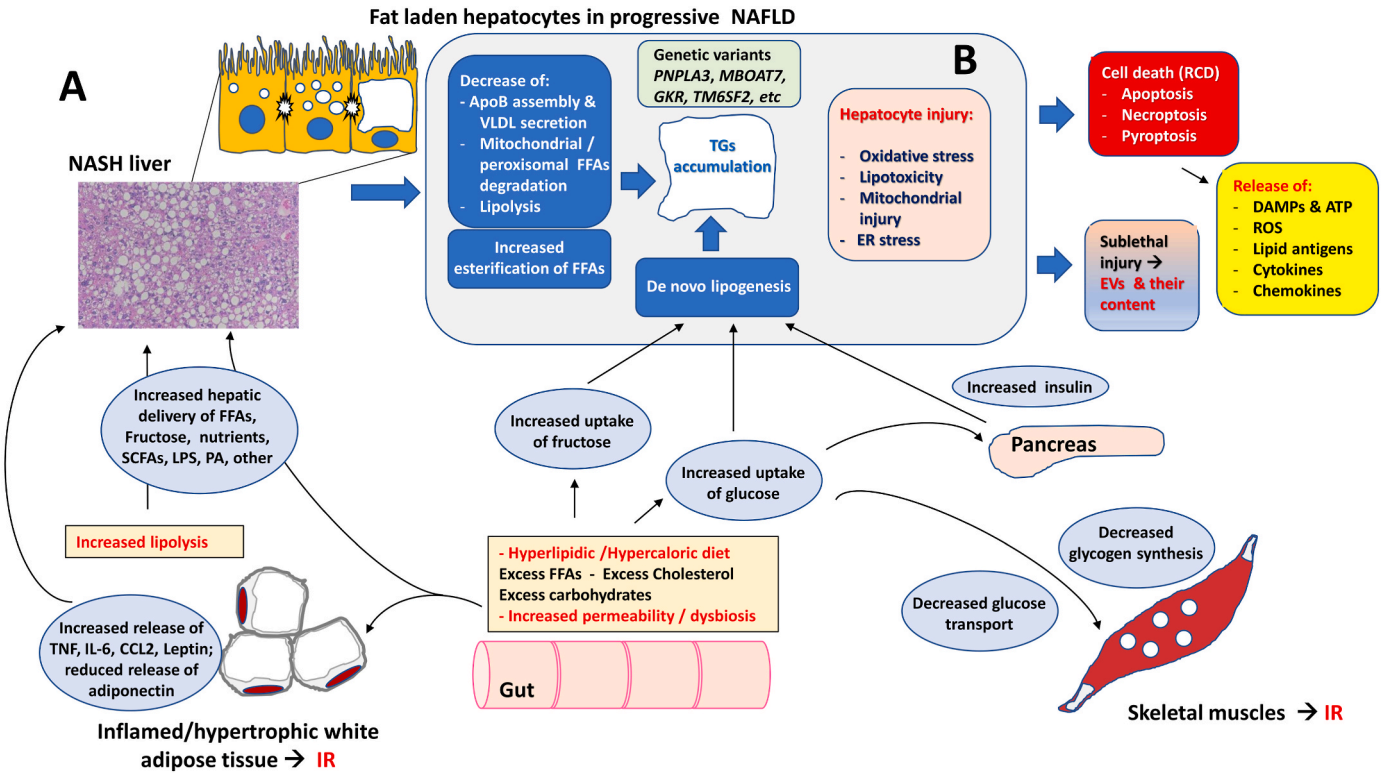

Fig. 1. The metabolic scenario of progressive NAFLD. (A) NAFLD and its progression are intimately related to an increased flux to the liver compartment of free-fatty acids (FFAs), carbohydrates and other nutrients as well as mediators and molecules coming from inflamed and hyperthrophic white adipose tissue (WAT) as well as from gut, the latter due to hyperlipidic/hypercaloric diet and to gut increased permeability and dysbiosis. **(B)** As a consequence of the metabolic scenario, and also in relation to the raising condition of insulin resistance (IR), hepatocytes accumulate tryglycerides (TGs) for a number of reasons, including: i) a decrease of FFAs oxidative degradation; ii) a decrease in ApoB assembly and VLDL secretion; iii) *de novo* lipogenesis, due to increased uptake of glucose and fructose; iv) increased esterification of FFAs. Genetic variants for several genes can exacerbate the alteration in lipid metabolism and favour progression of the disease. Fat-laden hepatocytes can then undergo injury (mainly related to oxidative stress, lipotoxicity, ER stress and mitochondrial damage) and death mainly through apoptosis, necroptosis and pyroptosis. Sublethal injury can also lead hepatocytes to release extracellular vesicles (EVs).

increased flux of fatty acids towards the liver (discussed in: Loomba et al., 2021).

3.2. Hepatic dysregulation of lipid and carbohydrate metabolism in NAFLD

According to the previously mentioned issues and with a major role for increased delivery of fatty acids to the liver and the development of IR, in peripheral as well as hepatic tissues, the normal lipid and carbohydrate metabolism in hepatocytes becomes dysregulated in NAFLD patients (Tilg et al., 2017; Samuel and Shulman, 2018; Loomba et al., 2021). As synthetized in Fig. 1B, the following interconnected major events contribute to the development of NAFLD-related steatosis: i) increased *de novo* lipogenesis (DNL), which is resulting mainly from increased delivery to the liver of glucose and other carbohydrates, mainly fructose, being strictly associated with hyperinsulinemia; this leads, as a major event, to up-regulation of key genes encoding for lipogenic enzymes regulating DNL and operates mainly through the involvement of sterol regulatory element binding-protein 1c (SREBP1c) as well as of other pro-lipogenic transcription factors, including carbohydrate response element binding-protein (ChREBP) Liver X receptors (LXRs) or peroxisome proliferator-activated receptor γ (PPARγ); ii) increased esterification of fatty acids into TG, involving increased formation of diacylglycerol (DG) and increased activity of diacylglycerol O-acyltransferase 2 (DGAT2), as a result of increased delivery of fatty acids to the liver due to dietary supply and/or sustained lipolysis by WAT. Although hepatocytes respond to the increased FA delivery and TG formation, they progressively face several metabolic limitations which involve: i) a decreased intrahepatic lipolysis as well as, more relevant, ii) a decreased or insufficient TG export via VLDL, and iii) a decreased/insufficient fatty acid metabolism via mitochondrial and/or peroxisomal oxidation. Some of these events can be exacerbated in the single individual by variants of genes involved in lipid metabolism (see section 3.3). Moreover, the attempt to increase mitochondrial β-oxidation of free fatty acids (FFAs) is potentially deleterious: Coenzyme A (CoA)-linked FFAs are shuttled into the mitochondrial matrix through carnitine O-palmitoyltransferase (CPT)1 and CPT2 but this leads to an excess of acetyl-CoA into the tricarboxylic acid cycle and of NADH into the mitochondrial electron transport chain; such an increased mitochondrial oxidative metabolism, in turn, can lead to an exhaustion of superoxide dismutase (SOD) 2 and GSH-peroxidase activity, resulting in an increased production of reactive oxygen species (ROS) and then of oxidative stress.

3.3. Genetic causes of NAFLD

In the last two decades it has become evident that NAFLD progression towards NASH and more advanced stages of CLD, in addition of - or apart from mechanisms leading to hepatocyte injury and death (see section 4.1), is mostly occurring in patients carrying one or even more genetic variants of genes mostly encoding for proteins involved in lipid metabolism (Anstee et al., 2016; Eslam et al., 2018; Eslam and George, 2020). This issue is relevant since, in addition to the other known drivers of NASH progression (i.e., metabolic or environmental ones), the presence of specific genetic variants can further impact on the highly inter-patient variability typically identified in NAFLD/NASH patients (Arrese et al., 2021). Moreover, NAFLD by itself can be considered as an inheritable disease, based on epidemiological, familial aggregation and

twin studies, with hepatic steatosis and fibrosis having a highly significant shared gene effect (Schwimmer et al., 2009; Cui et al., 2016; Eslam et al., 2018). Although epigenetic factors may also offer an additional contribute, at least five genetic variants of genes have been characterized which are strongly associated with susceptibility to NAFLD and/or its progression. All these genetic variants refer to genes that encode for protein or enzymes involved in the control of hepatic lipid metabolism, including patatin-like phospholipase domain containing 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), membrane bound O-acyltransferase domain-containing 7 (MBOAT7), glucokinase regulator (GCKR) and hydroxysteroid 17β-dehydrogenase (HSD17B13) (Eslam et al., 2018; Eslam and George, 2020). Major issue on these five genetic variants can be summarized as follows: i) PNPLA3: the protein encoded by this gene is an enzyme displaying triacyl-glycerol lipase and acyl-glycerol O-acyltransferase activity (Jenkins et al., 2004; Bruschi et al., 2017) in hepatocytes but also retinyl ester activity in hepatic stellate cells (HSCs) (Pirazzi et al., 2014; Bruschi et al., 2017). The genetic variant implies in the enzyme the substitution of an isoleucine at position 148 with a methionine (rs738409 C > G encoding for PNPLA3 I148M); this variant was first identified in 2008 (Romeo et al., 2008) in the first genome-wide study (GWAS) performed in NAFLD patients. This loss of function genetic variant is the most strongly associated with the development of steatosis and fibrosis and its expression confers a ten folds increased risk to develop HCC (Sookoian et al., 2009; Valenti et al., 2010; Liu et al., 2014a). ii) TM6SF2: the function of the encoded protein is not completely understood but is believed to control cholesterol metabolism and secretion of lipoproteins (Mahdessian et al., 2014; Li et al., 2018b). The TM6SF2 rs58542926 C > T polymorphism predicts NASH progression and results in reduced transcription and synthesis of the protein being associated with a decrease in the risk for cardiovascular diseases (Li et al., 2014b; Anstee et al., 2016). iii) MBOAT7: the encoded protein is involved in the remodelling of phosphatidylinositol with arachidonic acid in the Lands cycle; the rs641738 C > T genetic variant, resulting in reduced levels of phosphatidyl-inositol containing arachidonic acid in both hepatocytes and blood, has been associated with the risk to developing progressive NAFLD/NASH as well as HCC (Mancina M et al., 2016; Luukkonen et al., 2016; Donati et al., 2017). iv) GCKR: the protein GCKR is controlling *de novo* lipogenesis by regulating glucose uptake by hepatocytes; a genetic variant of GCKR gene encodes for a protein variant that modulates glucokinase in response to fructose-6-phosphate: this results in an increase of substrates available for fatty acid synthesis, then accelerating the lipogenic pathway and increasing the risk for NAFLD (Valenti et al., 2012; Eslam et al., 2018). v) HSD17B13: the normal HSD17B13 gene encodes for an enzyme displaying retinol dehydrogenase activity that localises specifically to hepatocytes' lipid droplets; a loss-of-function genetic variant of this gene, which generates a truncated and inactive form of the enzyme, has been reported to be associated with a protection from hepatic inflammation, fibrosis and HCC development in both alcohol-related liver disease (ALD) and NAFLD/NASH patients (Abul-Husn et al., 2018). This means that the "normal" enzyme, which is up-regulated in NASH patients, can significantly contribute to the disease.

A more detailed analysis of the relationships between the increasing list of genetic variants, the susceptibility to NAFLD and the progression of the disease can be found in authoritative reviews (Eslam et al., 2018; Eslam and George, 2020; Trépo and Valenti, 2020).

3.4. The role of gut microbiota in the development of NAFLD/NASH

Changes in gut microbiome as well as gut dysbiosis, altered gut barrier function and overall altered gut-liver axis are known to play a relevant role in the pathogenesis of NAFLD (Loomba et al., 2021). Concerning gut microbiome, the association of NAFLD/NASH with changes in microbiota composition is now an established concept supported by several studies (reviewed in: Loomba et al., 2017; Marra and Svegliati Baroni, 2018; Caussy et al., 2019; Schwimmer et al., 2019; Loomba et al., 2021). During NAFLD progression it has been reported that the microbiome of patients is becoming less heterogeneous, being enriched in Streptococcus and Gram-negative bacteria (Hoyles et al., 2018) and with the prevalence of Bacteroidetes and Ruminococcus being, in turn, associated with steatohepatitis and fibrosis, respectively (Boursier et al., 2016). Moreover, these changes in NAFLD patients with advanced fibrosis, that also include a raise in other bacterial genera (like E. coli and proteobacteria), are usually accompanied by a significant decrease in Firmicutes (Loomba et al., 2021). Along these lines, studies performed on well characterized cohorts of NAFLD patients have disclosed that a gut microbiome – based metagenomic signature may discriminate patients carrying mild to moderate fibrosis from those with advanced fibrosis (Loomba et al., 2017) or that a microbiome signature may specifically detect NAFLD-related cirrhosis (Caussy et al., 2019). However, due also to the influence of host and environmental factors on the gut microbiome (Li et al., 2014), it is unclear whether these signatures may have an overall diagnostic value. Moreover, it is also uncertain whether microbiome changes may be causally associated with fibrogenic progression or represent its consequence.

The contribution of changes in gut microbiota to NAFLD is likely to originate from multiple mechanisms, including at least the following (reviewed in more details in: Marra and Svegliati-Baroni, 2018; Loomba et al., 2021): i) a consistent alteration of energy harvest and decreased microbial diversity; ii) increased circulating levels of bacterial products/endotoxins that, by binding toToll-like receptors (TLRs) in hepatic cells or, possibly, adipocytes, may trigger inflammatory response; moreover, microbial products may also affect the release of incretins, then potentially and indirectly affecting glucose metabolism; iii) the release of other microbial metabolites (ethanol, phenylacetic acid, branched-chain and aromatic amino acid levels) has been proposed to increase hepatic TG accumulation; iv) increased bacterial synthesis of short chain fatty acids (SCFAs) that may operate either as lipid precursors, possibly boosting lipogenesis and gluconeogenesis, as toxic lipids or as ligands for G-protein coupled receptors; v) suppression of the production of ANGPTL4, resulting in increased lipid deposition in both hepatocytes and adipocytes; vi) dysregulation of gut-liver axis can affect bile acid levels which are known to be also metabolized by gut bacteria, then affecting pathways related to the binding of bile acids to farnesoid-X receptors (FXRs) in hepatocytes (i.e., affecting lipid metabolism) and in enterocytes (affecting lipogenesis and gluconeogenesis via release of FGF15/19 and FGF4 complexed to β-klotho).

4. Hepatocyte injury and death in NAFLD/NASH

4.1. Hepatocyte injury in progressive NAFLD: of lipotoxicity, oxidative stress and mitochondrial dysfunction

Although the presence of fatty liver is the most common feature of NAFLD patients, there is a substantial agreement on the fact that steatosis by itself, initially considered as a risk factor for NASH and fibrosis according to the 2-hits hypothesis (Day and James, 1998), is essentially a rather benign condition and that hepatocyte injury mostly depend on lipotoxicity, oxidative stress, mitochondrial dysfunction and additional mechanisms and processes but not on TG accumulation (Neuschwander-Tetri, 2010; Marra and Svegliati-Baroni, 2018; Loomba et al., 2021). Experimental studies in murine models of NASH have documented that the attempt to block TG synthesis by employing anti-sense nucleotides for the enzyme diacylglycerol acyltransferase 2 (DGAT2, which catalyzes fatty acid incorporation in TG, the final step in hepatocyte TG biosynthesis) was very efficient in reducing steatosis. However, this treatment at the same time increased hepatic FFAs, cytochrome P4502E1, markers of lipid peroxidation and of oxidative stress as well as lobular necroinflammation and fibrosis, then worsening the experimental scenario (Yu et al., 2005; Yamaguchi et al., 2007). In addition, TG accumulation is not sufficient to cause IR and inflammation in the liver, as shown in mice genetically manipulated to overexpress

DGAT2 (Monetti et al., 2007). This suggests that TG accumulation may potentially represent by itself a protective mechanism to prevent NAFLD progression (Yamaguchi et al., 2007; Marra and Svegliati Baroni, 2018).

Concerning the role of lipotoxicity, during the course of NAFLD/NASH progression hepatocytes are likely to face an overabundant delivery of lipid substrates as well as an impairment of lipid-related metabolic pathways (i.e., mitochondrial or peroxisomal β-oxidation, transient store of excess lipid as inert TG, secretion of TG via VLDL). These two aspects are believed to generate lipotoxic lipids resulting in mitochondrial dysfunction, ER stress and oxidative stress, eventually leading to hepatocyte injury and death, genomic instability, persistent inflammation, and chronic activation of fibrogenesis (Neuschwander-Tetri, 2010; Marra and Lotersztajn, 2013; Marra and Svegliati-Baroni, 2018; Schuster et al., 2018; Loomba et al., 2021). Along these lines, the list of major toxic lipids includes monounsaturated fatty acids, ceramides, lysophosphatidyl choline, lysophosphatidic acid and diacylglycerols. Monounsaturated fatty acids like palmitate (C:16) and stearate (C:18) are directly cytotoxic and able to induce apoptosis by activating c-Jun-N-terminal kinases (JNKs) and mitochondrial death pathways as well as by eliciting ER stress and promoting the activation of inflammasome (Marra and Svegliati-Baroni, 2018; Schuster et al., 2018). Free cholesterol can exacerbate cell injury mediated by death signals (TNF, TAIL, etc) and, following accumulation in hepatocytes as well as other cells (Kupffer cells or KCs, HSCs), can lead to disruption of membrane fluidity, oxidative stress, mitochondrial dysfunction, and ATP depletion, eventually leading to either apoptosis of necrotic-like cell death (Schuster et al., 2018). Of interest, cholesterol crystals have been reported to form on the lipid droplet membrane of hepatocytes and to cause activation and cholesterol loading of KCs and macrophages that surround and process these lipid droplets of dead hepatocytes (Ioannou et al., 2017). Moreover, lipotoxicity has been reported to cause the release of extracellular vesicles (EVs) from fat-laden hepatocytes that, in turn, can exert multiple effects on surrounding cells displaying pro-angiogenic and pro-inflammatory effects, inducing up-regulation of NOD-like receptor protein 3 (NLRP3) inflammasome and direct profibrogenic effects on HSCs (Povero et al., 2013, 2015; Hirsova et al., 2016; Cannito et al., 2017b).

Oxidative stress is another factor significantly contributing to hepatocyte injury during NAFLD fibrogenic progression, as already mentioned in this review, with the extent of oxidative stress and of its markers correlating with liver damage in patients (Ikura et al., 2006; Novo and Parola, 2008; Nobili et al., 2010; Higashi et al., 2017; Cannito et al., 2017a; Parola and Pinzani, 2019; Novo et al., 2020; Schwabe et al., 2020; Bocca et al., 2022). Several exhaustive reviews on the role of ROS and oxidative stress in liver fibrosis, including NAFLD/NASH, are available in the literature (Parola and Robino, 2001; Novo and Parola, 2008; Parola et al., 2008; Leung and Nieto, 2013; Paik et al., 2014; Cannito et al., 2017a; Bocca et al., 2022) and here only few selected major aspects and concepts will be recalled: i) Oxidative stress *per se* can contribute to hepatocyte injury and death, as well as to inhibit hepatocyte proliferation, then favouring the perpetuation of hepatic injury and inflammatory response; however, the generation of ROS and other oxidative stress – related intermediates, including reactive nitrogen species (RNS) and aldehydes from lipid peroxidation like 4-hydroxy-2,3-alkenals, is involved in almost all mechanisms of injury and processes (see next section) leading to hepatocyte death; ii) ROS generation in injured liver is mostly related to activation of innate immunity cells like KCs, recruited macrophages and neutrophils, including activation by damage-associated molecular patterns (DAMPs), with a particular role for NADPH-oxidase activation in several liver cells, also following the interaction of growth factors, cytokines, chemokine and other mediators with their cognate receptors; iii) ROS generation also relies on mitochondrial dysfunction as well as, specifically in alcoholic hepatitis and NASH, up-regulation of cytochrome P450 2E1 isoform; in particular, under conditions of NASH-related ER stress, ROS are generated at high levels by dysfunctional mitochondria as a consequence of ER leakage of Ca2+ that, once taken up by mitochondria, leads to the opening of MPT pores and derangement of mitochondrial electron chain; iv) ROS and 4-hydroxy-2,3-alkenals like HNE (4-hydroxy-2,3-nonenal) are able to directly exert a pro-fibrogenic action on HSCs and liver myofibroblasts (MFs), by stimulating for example the synthesis of ECM components but also mediating in these cells chemotactic effects exerted by a number of cytokines, growth factors and chemokines (Zamara E et al., 2004; Novo et al., 2006; Novo et al., 2011).

Concerning mitochondrial dysfunction, we have already mentioned its pivotal role particularly in the transition between simple steatosis to NASH, with fatty acid oxidation and lipotoxicity representing the major drivers of mitochondrial alterations. Once again, an increased flux of FFAs through mitochondria can lead to increased ROS generation able to damage mitochondrial DNA (mtDNA) and the protein complexes within the respiratory chain. In these metabolically altered conditions mitochondria become progressively more dysfunctional, then exacerbating oxidative stress but also leading to ATP depletion, loss of mitochondrial integrity and eventually cell death (Luedde et al., 2014; Machado and Diehl, 2016; Schuster et al., 2018). Interestingly, in the scenario of NASH-related apoptosis, oxidized mtDNA released in the cytosol has been reported to activate NLRP3 inflammasome (Shimada et al., 2012; Carlos et al., 2017).

4.1.1. Hepatocyte death in NAFLD/NASH: apoptosis, necroptosis, pyroptosis and autophagy

In the histopathological spectrum of NAFLD, in addition to steatosis, inflammatory infiltrate and fibrosis, NASH is characterized by significant loss of hepatocytes as well as by ballooning degeneration, a rather peculiar form of sub-lethal cell injury. Interestingly, ballooned cells, also referred to as "*undead cells*", are injured but still living hepatocytes with a characteristic morphology; these ballooned cells have been reported to exacerbate inflammatory and fibrogenic signalling in the surrounding microenvironment (Ibrahim et al., 2018). Concerning true parenchymal cell death, it has been reported that during NASH hepatocytes can dye because of three major variants of regulated cell death (RCD), including apoptosis, necroptosis and pyroptosis (Luedde et al., 2014; Hirsova and Gores, 2015; Schuster et al., 2018; Knorr et al., 2022). Although at present the relative contribution of the different types of cell death has not been clearly outlined, these model processes can use different mechanisms to trigger cell death, including or not the hepatocyte release of DAMPs, then exerting different roles in NAFLD progression. Moreover, hepatocyte death and inflammatory response are intimately related and can positively modulate each other. According to the overwhelming literature on this specific topic (reviewed in: Luedde et al., 2014; Hirsova and Gores, 2015; Schuster et al., 2018; Knorr et al., 2022), several major critical aspects and issues can be briefly summarized for each of the model processes.

Concerning apoptosis, this type of cell death is considered as critical for progressive NAFLD in patients and may be triggered by different stimuli, factors, or conditions. This includes lipotoxicity that, following an increased delivery of FFAs to the liver, can induce permeabilization of lysosomes, mitochondria dysfunction and apoptosis (Feldstein et al., 2004; Yamaguchi et al., 2007; Hirsova et al., 2016), with a suggested NASH-related role for transmembrane BAX inhibitor motif-containing 1 (TMBIM1), a protein regulating the multivesicular body (MVB)-lysosomal pathway whose expression can suppress steatohepatis in a murine dietary model of NASH (Zhao et al., 2017). In addition, pro-apoptotic hyper-activation of apoptosis signal-regulating kinase 1 (ASK1), has been detected either in patients with NASH or in murine models of NASH (Xiang et al., 2016; Wang et al., 2017). This is relevant since ASK1 is known to activate JNK and p38 MAPK pathways in response to ROS, ER stress, LPS and Ca^{2+} overload, then potentially contributing to hepatocyte death, inflammation and liver steatosis (Ha et al., 2009; Xiang et al., 2016). ASK1-mediated apoptosis is also known to be ameliorated by the deubiquitinating enzyme TNF-α induced protein 3 or TNFAIP3 (Zhang et al., 2018). Moreover, a drug mimicking Fas-associated death

domain (FADD)-like apoptosis modulator (CFLAR) has been reported to block ASK-1 dimerization and to inhibit experimental NASH in mice and in non-human primates (Wang et al., 2017). Another factor contributing to hepatocyte apoptosis is represented by caspase 2, a proteolytic enzyme able to activate site 1 protease (S1P) which is induced by either TNFα or ER stress in experimental NASH. In particular, both pharmacological inhibition and genetical deletion of caspase 2 have been reported to strongly inhibit experimental NASH (Machado et al., 2015; Kim et al., 2018).

Concerning the role of necroptosis in progressive NAFLD, this form of cell death is a variant of RCD induced by death signals (TNFα, FasL, TNF-related apoptosis-inducing ligand-1 or TRAIL-1, etc.) that requires the availability of active receptor-interacting protein kinase 1 (RIPK1) and, particularly, RIPK3 and operates in conditions in which initiating caspases (caspase 8 or 10, murine or human, respectively) are inactivated (Schwabe and Luedde, 2018). In these conditions the ligand receptor pathway leads to the assembly of the necrosome complex, the recruitment of a pseudokinase, known as mixed-lineage kinase domain-like protein (MLK1). The necrosome, particularly through MLK1, is recruited to plasmamembrane and is responsible for a lytic form of cell death leading to hepatocyte swelling and membrane rupture, then originating DAMPs able to sustain inflammatory response. It should be noted that RIPK3 has been reported to also activate the NLRP3 inflammasome (Lawlor et al., 2015) and that inhibition of RIPK3 can attenuate liver injury and fibrosis in the methionine and choline – deficient (MCD) diet murine model of NASH (Gautheron et al., 2014).

Other selected critical aspects potentially related to inflammatory and fibrogenic response can be summarized as follows: i) murine studies have revealed the mechanistic relevance of pro-apoptotic signalling such as those represented by the interaction of TRAIL with its cognate receptors (Idrissova et al., 2015; Hirsova et al., 2017); moreover, pro-apoptotic TRAIL-mediated signalling can also promote the release of EVs and several chemokines, resulting in recruitment and activation of immune cells (Hirsova and Gores, 2015); ii) KCs, following significant phagocytosis (i.e., engulfment) of hepatocellular apoptotic bodies, become hyper-activated and up-regulate expression and release of TNF and FasL (Canbay et al., 2003a), then promoting further hepatocellular apoptosis in an overall pro-inflammatory and pro-fibrogenic manner; iii) hepatocytes undergoing apoptosis have been reported to release apoptotic bodies and fragments of DNA that can be taken-up by HSCs stimulating their activation and phenotypic pro-fibrogenic responses (Canbay et al., 2003b; Watanabe et al., 2007).

Pyroptosis is another variant of RCD which has been more recently involved in the pathogenesis of progressive NAFLD (Knorr et al., 2022). Pyroptosis has been defined as an inflammatory-related cell death originally described as a way for phagocytes to clear intracellular pathogens involving cell lysis as well as excess production and release of IL-1β and IL-18 to then exacerbate immune response (Zychlinsky et al., 1992; Brennan and Cookson, 2000). At present we know that pyroptosis affect several cell types, including hepatocytes, and operates through the proteolytic cleavage of proteins defined as gasdermin D (GSDMD) or, for certain proteases, gasdermin E (GSDME). In the conventional pyroptosis the cleavage leads to the generation of N-terminal fragments of these proteins referred to as GSDMD-N or GSDME-N which oligomerize at plasma membrane level inducing the formation of pores inducing cell swelling, mitochondrial membrane depolarization, chromatin condensation eventually leading to membrane rupture, release of DAMPs and of IL-1β and IL-18 (Kayagaki et al., 2015; Shi et al., 2015). Several proteolytic mechanisms have been reported to induce formation of pores by oligomerization of either GSDMD-N fragments or of homologous fragments of gasdermin E, including the activation of NRLP3 inflammasome – dependent caspase 1 or the activation of other proteases such as caspase 11, caspase 4/5, caspase 3, cathepsin B as well as even neutrophil elastase and granzyme B (Broz et al., 2020; Knorr et al., 2022). Concerning the NLRP3 inflammasome, which is known to be involved in steatohepatitis, clinical and experimental studies have reported up-regulated expression of all components including NLRP3 itself, the protein ASC, caspase 1 as well as pro-IL-1β and pro-IL-18 (Wree et al., 2014; Gaul et al., 2021; Knorr et al., 2022). In particular, liver and circulating levels of active caspase 1 were significantly increased in NASH patients and this increase was correlated to the severity of the disease and the stage of fibrosis (Gaul et al., 2021). The activation of caspase 1 triggered by NLRP3 hyperactivation led to pyroptosis as well as to activation of HSCs (possibly by internalization of particles released by injured hepatocytes) and of their phenotypic responses (Gaul et al., 2021). Mechanistically, the role for GSDMD and then pyroptosis has been revealed though genetic depletion of this protein, resulting in a significant attenuation of experimental NASH associated to reduced inflammation as well as steatosis (Xu et al., 2018). Moreover, the N-terminal domain of GSDMD has been proposed also as a putative biomarker of steatohepatitis (Xu et al., 2018).

Dysregulation of autophagy is an additional mechanism that has been implicated in the pathogenesis of NASH and then in determining hepatocyte injury and death, although it may also affect macrophages and HSCs (Amir and Czaja, 2011; Czaja, 2016). This involvement is not in principle surprising since in hepatocytes a specific variant of autophagy, commonly defined as lipophagy, is known to contribute to degrade also lipids and then TGs (Singh et al., 2009). Moreover, autophagic proteolysis has been found to be significantly dysfunctional in NASH patients in association with a decreased inhepatic expression of cathepsins (B, D and L), as also confirmed by evidence for an increased aggregation of p62 (Fukuo et al., 2014). Although electron microscopy identified an increase of autolysosomes in the hepatocytes of NAFLD patients, supposed to be a consequence of steatosis, these data were suggesting that decreased cathepsin activity impairs degradation of proteins incorporated by autophagosomes and autophagic membranes, supporting the hypothesis that proteolytic activity of autophagy is inhibited through suppression of lysosomal enzyme activity in NAFLD (Fukuo et al., 2014). Moreover, hepatic inflammation correlated with autophagic dysfunction in NAFLD. Almost homologous data were obtained in murine genetic or dietary models of obesity (Yang et al., 2010), in which the dysfunctional autophagy has been related to the induction of ER stress, potentially leading to hepatocyte apoptosis, and insulin resistance (Yang et al., 2010). The link between dysfunctional autophagy and the induction of apoptosis has been confirmed by a study performed in genetically manipulated mice showing that hepatocyte-specific autophagy deficiency can indeed result in cell injury and apoptosis through the unfolded protein response. Moreover, the same study also suggested that dysfunction of mitophagy, which can eliminate altered/damaged mitochondria, may contribute to apoptosis in case the mitochondrial pro-apoptotic pathway was induced (Kwanten et al., 2016). In addition, it is well known that a deficient ability to degrade damaged mitochondria can result in oxidative stress as well as in the release of mitochondrial factors triggering intrinsic apoptosis (Lemasters, 2005). It should be noted that several mechanisms have been reported to down-regulate autophagy during progressive NAFLD, including impairment of the fusion of autophagosomes with lysosomes as well as decreased expression of autophagy-related genes or reduced levels of lysosomal enzymes (Amir and Czaja, 2011).

5. Innate and adaptive immunity in progressive NAFLD: a matter of cell-to-cell interactions, mediators, and immune-related mechanisms

Dysregulated metabolism and hepatocyte injury are key drivers of NAFLD/NASH, but a major role in NAFLD progression is played by multiple other hepatic and extrahepatic cell lineages within the liver fibrotic microenvironment. These non-parenchymal cells are responsible for the perpetuation of inflammatory response, activation of mesenchymal cells (mainly HSCs and portal fibroblasts) to myofibroblast-like cells, excess deposition of ECM components and, where possible, fibrosis resolution. Taking advantage from novel

technological approaches we will analyse established and emerging roles for these cell lineages with a focus on innate immunity cells (KCs and macrophages recruited from peripheral blood), adaptive immunity cells (lymphocytes, NK and NKT cells) and hepatic MFs as well as on cell-to-cell interactions in terms of molecular mediators and signalling pathways involved (Trautwein et al., 2015; Higashi et al., 2017; Cannito et al., 2017a; Tacke, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Carter and Friedman, 2022; Peiseler et al., 2022; Wallace et al., 2022).

5.1. The impact of novel technologies in outlining cellular interactions

Due to the complexity of the inflammatory/fibrotic microenvironment, a mandatory goal in this field of research has been to reach a comprehensive understanding of the *in vivo* interactions between the cell lineages involved. This is not in principle an easy task since during the progression of NAFLD/NASH one should consider that cell lineages involvement and cell-to-cell interactions are likely to be stage-specific and strictly related to the actual heterogeneous topography of hepatic microarchitecture that can include areas of hepatocellular proliferation alongside areas of unresolved inflammation and fibrogenesis. In the last two decades co-cultures of liver cells, hepatic spheroids as well as precision-cut liver slices and organoids, assisted or not by microfluidic apparatus, have been used to start to investigate cell-to-cell interactions carrying either advantages and disadvantages (for a recent and exhaustive review on this topic see Kaur et al., 2023).

On these premises and considering the pathophysiological complexity of progressive NAFLD, the conventional experimental approach employing two-dimensional (2D) co-cultures, although potentially useful, suffers of obvious limitations, including the use of a limited number of cell types (usually no more than two, for example hepatocytes and MFs or macrophages and MFs) and the absent reproduction of the *in vivo* microenvironment (van Grunsven, 2017). A certain degree of improvement has been obtained using 3D hepatic spheroids which can be produced and cultured through a wide variety of techniques and materials, often commercially available (van Grunsven, 2017; Mazza et al., 2017; Pingitore et al., 2019). It has been reported that these 3D spheroids could also incorporate multiple non-parenchymal cells (NPCs) like HSCs and KCs (Bell et al., 2016) and that can be used to test drug-induced liver injury and fibrosis (Bell et al., 2016; Leite et al., 2016) as well as steatosis (Pingitore et al., 2019). Other 3D models include also scaffold/matrix-based 3D cultures or, more recently, decellularized 3D scaffolds (Mazza et al., 2017; Thanapirom et al., 2021). The use of the approach of precision-cut liver slices has the advantage to maintain the original 3D liver architecture but the disadvantage to lack flow conditions usually granted by liver sinusoids in terms of mediators delivered from extrahepatic tissues. These limitations have been just partially overcome using perfused liver-on-a-chip models which ideally include a higher-throughput system able of mimicking hepatocytes conditions and the dynamic physicochemical hepatic environment (Hassan et al., 2020). A prototype device has been proposed being formed by a microtissue platform integrating NPCs in a vascular layer made of human umbilical vein endothelial cells (HUVECs) and KCs, and an opposing hepatic layer made of HSCs co-cultured with the hepatocyte cell line HepaRG (Rennert et al., 2015). More recently, a novel platform has been described that seems of specific interest. This procedure has been developed to generate multi-cellular human liver organoids employing induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). These organoids were composed of hepatocyte, HSCs as well as KCs and exhibited transcriptomic signature resembling in vivo-derived tissues. In particular, when these organoids were treated with fatty acids, they progressively developed key features of NASH, including steatosis, inflammation and fibrosis (Ouchi et al., 2019). A considerable technological breakthrough for determining cell-to-cell interactions has been afforded in the last four-five years using single-cell RNA-sequencing (scRNAseq) (Armingol et al., 2021). scRNAseq has been recently applied to resolve cellular heterogeneity and identify subpopulations of cells, particularly inflammatory cells, in either normal or chronically injured livers, including NASH livers, and some of the most interesting and recent finding will be recalled in the next sections (Krenkel et al., 2019; Xiong et al., 2019; Wang et al., 2021; Barreby et al., 2022; Hundertmark et al., 2022; Peiseler et al., 2022). scRNAseq analyses, which are quite efficient in capturing mRNAs in any cells, are based on the expression ligand-receptor pairs and repositories for these interactions. The analysis of cell-to-cell interactions within a normal or NASH liver requires multiple computational tools and associated ligand-receptor interaction databases which are now available in platforms like ICELLNET, CellPhoneDB and CellChat. The only problem is represented by the fact that these platforms are not still sufficiently integrating the spatial context of these interactions: the integration of spatial transcriptomics into these platforms should allow to better assess communication between different cells in a defined tissue. Moreover, these analyses are unable to capture ligands like lipids or bile acids and should be also integrated by proteomics since transcriptomics may not accurately reflect protein expression (Peiseler et al., 2022).

A final mention in this paragraph is for the emerging potential of topological analysis of tissue microenvironment by means of imaging mass cytometry or IMC (Giesen et al., 2014; Schapiro et al., 2017). The available IMC systems are unique in allowing to visualize simultaneously several protein markers in desired tissues and tumors at subcellular resolution but preserving the information related to tissue architecture and cell morphology to likely uncover new biomarker correlations and cell-to-cell interactions. IMC is a technique that has only recently applied to liver diseases, at present mainly for studying the microenvironment of HCC (Leslie et al., 2022; Sheng et al., 2022), which has already revealed a high degree of heterogeneity within the tumour microenvironment and intratumoural region-specific distributions of immune cells.

5.2. Inflammatory and immune responses in NAFLD/NASH: some key general concepts

The use of novel technologies to investigate clinical and experimental conditions of NASH, including scRNAseq and single cell multiomics, spatial transcriptomics, IMC, intravital microscopy and others, has provided evidence that the immune cell composition is definitively reshaped during steatohepatitis (Davis et al., 2019; Ramachandran et al., 2020; Saviano et al., 2020; Guilliams et al., 2022). Moreover, NAFLD-associated inflammation is reflecting systemic changes being sustained by multiple conditions and organ systems as well as by multiple intrahepatic and extrahepatic factors, with inflammatory signals coming from adipose tissue, gut, skeletal muscle, and bone marrow. As described in previous sections, the progression from simple steatosis to steatohepatitis involves metabolic dysfunctions, lipotoxicity, oxidative stress and eventually hepatocyte injury and death, either via apoptosis or necroptosis or pyroptosis. All these events and processes are responsible for sustaining a persistent inflammatory response and chronic activation of repair mechanisms that involve several innate and adaptive immunity cells and the contribution of these cells will be analysed in the following sections. In relation to hepatocyte injury and death, this critical event can initiate inflammation through several ways. Injured hepatocytes of course can release damage-associated molecular patterns (DAMPs), like ATP, DNA fragments, histones, and others, that can activate pattern recognition receptors (PRRs) and then innate immunity cells (Gong et al., 2020). Injured cells can also directly release pro-inflammatory cytokines like IL-1β and IL-18, following inflammasome activation (Alegre et al., 2017; Wang et al., 2021) as well as indirectly, particularly fat laden hepatocytes, through the release of EVs containing the C-X-C motif chemokine ligand (CXCL)-10, mitochondrial DNA as well as several other mediators (Ibrahim et al., 2016; Cannito et al., 2017b) that can be taken up by surrounding cells.

According to current literature, innate immune mechanisms and

related cells are believed to play a major role in sustaining inflammatory response in NASH progression (Peiseler et al., 2022; Wallace et al., 2022), with a focus on the role of macrophages (see next section). However, growing evidence also supports a critical contribution by adaptive immunity (Sutti and Albano, 2020) and this is not surprising since immune cells in normal conditions have a role in preserving an immunotolerant microenvironment which is fundamental since the liver, through portal circulation, is continuously exposed to bacterial products and diet-derived antigens (Heymann and Tacke, 2016; Portincasa et al., 2021). The mentioned metabolic alterations (increased flux of FFA to the liver, lipotoxicity, oxidative stress, etc.) combined with gut dysbiosis (that means abnormal growth of harmful bacterial strains able to induce increased permeability of gut mucosal barrier and translocation of bacterial products or PAMPs) can trigger inflammatory response, sustain hepatocyte death and subvert the immunotolerant hepatic environment (Vallianou et al., 2021; Hughey et al., 2022) but also play a major role in activating HSCs and portal fibroblasts to hepatic MFs (Koyama and Brenner, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Carter and Friedman, 2022). An additional contribution able to sustain hepatic inflammation may be provided by platelets, which have been reported to significantly infiltrate the injured livers in both murine studies and in NASH patients (Malehmir et al., 2019; Ramadori et al., 2019). Interestingly, a correlation between mean platelet volume (MPV, an index of increase platelet production) and the degree of inflammation and fibrosis has been described in NAFLD/NASH patients (Alkhouri et al., 2012). Along these lines, following tissue injury, platelets undergo rapid activation and can release a number of inflammatory and pro-fibrogenic mediators, including TNFα, IL-6, transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), endothelial growth factor (EGF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor A (VEGF-A), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF) (Heijnen and van der Sluijs, 2015; Taus et al., 2019), as well as IL1β-loaded microparticles (Brown and McIntyre, 2011).

A final mention in this section concerns the involvement of biliary compartment in driving inflammation in NAFLD/NASH patients. In the liver of NASH patients is often detectable the "ductular reaction" (i.e., proliferation of biliary epithelial cells due to pre-existing cholangiocytes, hepatic progenitor cells or, still matter of discussion, transdifferentiation of hepatocytes into cholangiocytes) that correlates with portal inflammation, NASH activity score and fibrosis (Richardson et al., 2007; Zhou et al., 2021; Cadamuro et al., 2022). Reactive cholangiocytes or reactive ductular cells (RDC), following interactions with surrounding cells, have been described to contribute to both inflammation and fibrogenesis. For example, RDC have been reported to release proinflammatory mediators like IL-1, TNFα, IL-6, IL-8, C–C motif chemokine ligand (CCL) 2, CXCL10 and CXCL12, as well as mediators able to affect profibrogenic and other cells, including PDGF, TGFβ, VEGF-A and –C, connective tissue growth factor (CTGF) (Banales et al., 2019; Novo et al., 2020; Cadamuro et al., 2022).

5.2.1. The role of liver macrophages in NAFLD/NASH: of macrophage heterogeneity and of their interactions with other cell types

Experimental and clinical studies have pointed out the extreme heterogeneity of macrophages and of related subpopulations during conditions of progressive NAFLD, with KCs (i.e., the resident liver macrophages) and monocyte/macrophages recruited from peripheral blood, referred to as either monocyte-derived macrophages (MoMs) or as bone marrow - derived macrophages (BMDMs), playing a major role (Peiseler et al., 2022; Wallace et al., 2022). Here we will offer a synthesis of novel and critical data, often emerged by applying scRNAseq and other advanced technologies. 1) Liver KCs are believed to be mostly involved in the early phases of NAFLD progression by releasing major pro-inflammatory mediators like TNFα, IL-1β and CCL2, with KCs depletion during early experimental NASH reported to strongly attenuate inflammatory response and the overall severity of liver injury (Huang et al., 2010; Pan et al., 2018); KCs activation is elicited by DAMPs and, more specifically, by FFAs through NLRP3-inflammasome activation (Pan et al., 2018) and, related to gut dysbiosis, by LPS though TLR4 (Csak et al., 2011). It is interesting to note that murine KCs in normal liver represent a homogenous lineage positive for markers like F4/80, C-type lectin domain family 4 member F (CLEC4F) and T cell immunoglobulin and mucin domain-containing 4 (Timd4). By contrast, in human healthy liver monocytes/macrophages form a single population continuum, preventing the identification of a single population of KCs, as reported by a spatial proteogenomic cell atlas (Guilliams et al., 2022). However, using scRNAseq, human liver macrophages have been found to form two clusters of macrophages: i) a $CD68^{+}MARCO^{+}Timd4^{+}$ subset, considered as KCs and characterized by expression of immune-tolerance genes and ii) a $CD68^{+}MARCO^{-}Timd4^{-}$ subset, likely MoMs, expressing higher levels of pro-inflammatory genes (MacParland et al., 2018; Ramachandran et al., 2019; Zhao et al., 2020). 2) In NASH-related livers an expansion of hepatic macrophages is observed as a consequence of increased recruitment of monocytes from peripheral blood leading to a distinct population of MoMs. In murine NASH livers this recruitment, detected mainly in periportal areas, originated two distinct subsets of cells identified as $CCR2^{high}Ly6C^{high}$ pro-inflammatory monocytes and $CX3CR1^{+}Ly6C^{low}$ patrolling monocytes (Guilliams et al., 2018). $CCR2^{+}$ macrophages have a role in liver injury and pharmacological inhibition of their recruitment ameliorated insulin resistance, hepatic inflammation and fibrosis (Krenkel et al., 2018). In humans, recruitment of circulating monocytes leads to differentiation of three subsets, classical $CD14^{high}CD16^{neg}$ monocytes as well as intermediate $CD14^{high}CD16^{low}$ or non-classical $CD14^{low}CD16^{high}$ cells (Guillot and Tacke, 2019). Interestingly, even in patients with NASH it has been reported periportal accumulation of $CCR2^{+}$ inflammatory macrophages and this was found to correlate with NASH severity and fibrosis (Krenkel et al., 2018). 3) During the progression of NASH, KCs are gradually lost (possibly because of excess lipid uptake and storage gradually impairing their self-renewal ability) and experimental studies indicate that they were replaced by more pro-inflammatory and cytotoxic $Timd4^{neg}$ monocyte-derived KCs (Seidman et al., 2020; Tran et al., 2020), a scenario resembling the one recently detected also in humans (Guilliams et al., 2022). Moreover, scRNAseq and proteomic analyses have recently identified two distinct subsets of KCs in murine liver, defined as KC1 and KC2: KC1 cells showed low levels of CD206 and were negative for endothelial cell adhesion molecule (ESAM), whereas KC2 cells were $CD206^{high}ESAM^{+}$ and proposed to be less tolerogenic and to promote steatosis and steatohepatitis during obesity and TGs accumulation (Blériot et al., 2021). 4) In recent years a further and somewhat peculiar macrophage subset has been identified in fibrotic and cirrhotic livers on the basis of the expression of CD9 and of triggering receptor expressed on myeloid cells −2 (TREM-2) (Ramachandran et al., 2019). These $CD9^{+}TREM2^{+}$ cells, isolated using flow cytometry and then analysed with scRNAseq, were initially defined as "*scar-associated macrophages*" (SAMs) and, because of their localization in the fibrotic niche, considered as pro-fibrogenic cells for their gene signature and the ability to activate HSCs in culture. Proteogenomic analyses also revealed that $CD9^{+}TREM2^{+}$ cells were detectable also in healthy livers and termed "*lipid-associated macrophages*" (LAMs) in both human and mice (Guilliams et al., 2022). These LAMs in physiological conditions were detected in periportal areas and in close connection with bile ducts, whereas in steatotic livers they increased in number and accumulated pericentrally (Guilliams et al., 2022). This means that MoMs recruited in the liver during progressive NAFLD have two distinct fate: to originate either monocyte-derived KCs (i.e., to replace lost KCs) or LAMs. At present the specific signals from liver microenvironment that may drive MoMs into one of the two options are only partially identified and the function of LAMs is still poorly understood but data indicating that LAMs are preferentially recruited to steatotic liver regions suggest that LAMs are induced by local lipid exposure (Guilliams et al., 2022). In addition, we know that LAMs express high levels of osteopontin (Remmerie et al.,

2020), known to be up-regulated in either murine or human NASH (Glass et al., 2018; Honda et al., 2020), and have been suggested to form the so-called "crown-like structures" (Olona et al., 2021) which are $CD11^{+}$ macrophages aggregated to surround hepatocytes with large lipid droplets (Itoh et al., 2013). Indeed, the loss of LAMs prevented the formation of "crown-like structures" but, unexpectedly, was associated with increased fibrosis in a dietary murine model of NASH (Daemen et al., 2021). Moreover, TREM2+ deficient mice develop an accelerated form of experimental NASH (Hou et al., 2021). LAMs and SAMs, which are likely to represent the same cell type and are detected also in adipose tissue of obese humans and mice, may have a regulatory role on energy supply/metabolic homeostasis rather than a role favouring progression of the disease (Jaitin et al., 2019). 5) Taking in mind the heterogeneity just described, hepatic macrophages have an obvious role in sensing metabolic injury and sustaining both chronic inflammation and liver fibrogenesis. These innate immunity cells, as well as MoMs or BMDMs, have been reported to interact with almost all the cells involved in the inflammatory and profibrogenic NASH liver environment, either intrahepatic as well as extrahepatic, as recently and extensively reviewed elsewhere (Lee and Friedman, 2022; Peiseler et al., 2022; Wallace et al., 2022). Concerning KCs, their NASH-related dysregulation can lead to DAMPs-mediated, TLR- and/or NF-kB-related increased production and release of several cytokines like TNFα and IL-6 but also IL-1β and IL-18 through activation of NLRP3 inflammasome, chemokines (mainly CCL2), eventually recruiting large amount of $CCR2^{high}Ly6C^{high}$ monocytes (Ramachandran et al., 2012) but also other innate immunity cells (like neutrophils). These cells can also interact with HSCs and/or MFs by releasing pro-fibrogenic growth factors (including TGFβ1). Whether MoMs or BMDMs are concerned, transcriptomic studies showed that in NASH livers they remain in a more pro-inflammatory state ($Ly6C^{high}$ and expressing IL-1β, TNFα, CCL2, etc.), sharing similarities with macrophages of adipose tissue and bone marrow (Jaitin et al., 2019; Krenkel et al., 2020), including the previously mentioned subset of SAMs or LAMs (Ramachandran et al., 2019). Although these MoMs may interact with any surrounding cell type, it is worth mentioning that they have priviledged interactions first with HSCs and MFs. The following major issues should be rapidly recalled: i) MoMs can stimulate HSCs and induce their activation-transdifferentiation into MFs by relasing mainly PDGF (both a mitogen and a chemoattractant) and TGFβ1 (enhancing deposition of ECM components) (Parola and Pinzani, 2019; Lee and Friedman, 2022; Peiseler et al., 2022); ii) MoMs can release several GFs, cytokines and chemokines, including those just mentioned, to sustain the MF-like phenotype and MFs survival, the latter through NF-kB (Pradere et al., 2013), with CCL2 but also VEGF and PDGF working as ROS-dependent chemoattractants (Novo et al., 2011; Pellicoro et al., 2014); iii) the use of the already mentioned high resolution transcriptomic data and of scRNAseq has opened the way to analyse in depth ligand-receptor cell-to-cell interactions; as an example, by analysing in this way the fibrotic niche it has been possible to identify a ligand receptor pair between SAMs expressing PDGF, IL-1β and tumor necrosis factor (ligand) superfamily member 12 (TNSF12) with their cognate receptors PDGF-R, IL-1βR and TNFS12A, respectively (Ramachandran et al., 2019); iv) a recent study has outlined an additional and more general concept by a multidimensional analysis performed on NAFLD/NASH and PSC patients; although some differences between the two conditions were evident (i.e., predominant myeloid cell accumulation in NASH vs predominant infiltration of immune cells in PSC), by correlating the disease stage Authors observed that both etiologies were presenting an intense aggregation $IBA1^{+}$ $CD16^{low}$ $CD163^{low}$ macrophages in non-parenchymal areas and in spatial proximity to ductular cells, with macrophages originating from bone marrow – derived monocytes, as deduced from related murine models (Guillot et al., 2023). Since loss of hepatocytes and increased ductular reaction are tightly associated monocyte-derived macrophage accumulation represent a common immunological feature of the progression of CLDs of different aetiology (i.e., NAFLD, PSC, PBC and ALD), Authors suggest that the involvement of $IBA1^{+}$ $CD16^{low}$ $CD163^{low}$ macrophages may represent key and common pathogenic drivers for CLD progression (Guillot et al., 2023); v) one should also at least mention a very recent study that, although not directly related to NAFLD/NASH, has outlined an additional concept on the progressive loose of KCs and recruitment of MoMs (Peiseler et al., 2023); these Authors suggest that the gradual loss of KC in advanced fibrosis may promote a reorganization of MoMs that can functionally compensate some of KC-related functions. In particular, they showed that recruited monocytes, by following the formation of collateral vessels bypassing sinusoids, formed what have been called as *KC-like syncytia*, possibily able to capture bacteria from the bloodstream (Peiseler et al., 2023). 6) Concerning the involvement of KCs and MoMs in NASH progression it is worth mentioning the recently emerged role of cyclic GMP-AMP synthase (cGAS) activator of interferon genes (STING) signalling in NAFLD (and other CLD of different aetiology) as an additional mechanism involved in the regulation of innate immunity (Chen et al., 2021). Indeed, STING expression is up-regulated in NASH patients (Luo et al., 2018) and the STING-IRF3 pathway, activated in hepatocytes by aberrant DNA (mtDNA following mitochondrial injury or damaged/fragmented DNA) and contributing to induction of hepatocyte apoptosis and metabolic derangement, up-regulates the inflammatory response and the release of NF-kB-dependent cytokines as well as of interferons (IFNs) (Qiao et al., 2018). In relation to the interaction with NASH hepatocytes, STING was found to be overexpressed in KCs and MoMs in the liver of patients with NASH; moreover, STING positive macrophages were significantly increased in livers from NASH patients with fibrosis and positively correlated with liver inflammation grade and fibrosis stage (Wang et al., 2020). The activation of cGAS-STING pathway in KCs and MoMs, involving activation of TBK1, JNK, and NF-kB, resulted in activation of macrophages producing TNF-α and IL-1β, which trigger inflammatory pathways in hepatocytes, as well as TGF-β1, the latter potentially sustaining HSCs and MFs profibrogenic role (Wang et al., 2020). 7) Liver macrophages have been reported to play also a resolution role in case of cessation of either acute injury or, more related to the present review, following the removal of the underlying etiological agent or condition leading to CLD. Fibrosis reversion has been indeed documented in patients by monitoring sequential liver biopsies in patients with a CLD of different aetiology (Kisseleva and Brenner, 2020; Rockey and Friedman, 2021), including NASH in case of loss of weight or following bariatric surgery (Dixon et al., 2004; Hafeez and Ahmed, 2013). It has been proposed that removing the underlying aetiological agent led to the suppression of pro-inflammatory or profibrogenic mediators by ending hepatocyte injury, decreasing the release of DAMPs as well as the phagocytosis of cell debris. These events as well as the involvement of autophagy, specifically in NASH (Lodder et al., 2015), and possibly of fractalkine or C-X_3-C motif chemokine ligand 1 (CX_3CL1) (Karlmark et al., 2010) are critical in switching macrophages to the resolution phenotype of Ly-$6C^{low}$ cells (also positive for CX3CR1, CD206 and arginase −1 and −2). Resolution macrophages are known to exert their role mainly, but not uniquely, by releasing anti-inflammatory cytokines (IL-10, IL-1 receptor antagonist -a or IL1Ra), resolution growth factors (VEGF-A, hepatocyte growth factor of HGF) and effective matrix metalloproteases (MMPs) like MMP9, MMP12 and MMP13 (Tacke, 2017), with the release of MMP9 and TRAIL also reported to have a role in promoting MFs apoptosis (Ramachandran et al., 2012). Unfortunately, still few data are available that are specifically related to NASH. Of course, fibrosis regression involves more events and mechanisms, including senescence and apoptosis of activated MF-like HSCs, the involvement of NK and NKT cells and much more, as recently and extensively reviewed by others (Kisseleva and Brenner, 2020; Rockey and Friedman, 2021). Critical events and mechanisms occurring during fibrosis regression are summarized in Fig. 3.

5.2.2. *The role of neutrophils in NASH*

Whether neutrophils are concerned, the role of these cells in CLD progression was quite neglected until few years ago when evidence of

their involvement as well as of neutrophil extracellular traps (NETs) and NETosis in different chronic inflammatory diseases was first consistently reviewed (Jorch and Kubes, 2017; Soehnlein et al., 2017; Peiseler and Kubes, 2019). NETosis is a peculiar form of cell death, first identified in neutrophils exposed to certain bacteria, in which the neutrophils extrude their nucleic acids (and factors contained in their granules) to trap bacteria which can favour their subsequent phagocytosis. Although the role of neutrophils in NAFLD/NASH is still incompletely understood, their detection in the inflammatory infiltrate of biopsies from human NASH livers was already reported several years ago (Rensen et al., 2009; Gadd et al., 2014) and also confirmed in the liver of murine models of NASH, particularly in early phases (van der Windt et al., 2018; Hwang et al., 2021). Further issues related to neutrophils emerged for human NASH, including the following: i) the presence of neutrophils in human NASH livers (and sometimes in experimental NASH) is likely to be related to a parallel increased expression of specific chemoattractants like IL-8 (also known as CXCL8), CXCL1 and CXCL11, particularly if referred to simple steatosis, as well as of cognate receptors (Bertola et al., 2010); ii) the presence of NETs markers was first detected in the serum of NASH patients (van der Windt et al., 2018) and, more recently, NETs decorated with IL-1β and IL-17A have been described in liver biopsies from NASH patients but not in patients with simple steatosis and found to be associated with with inflammation, ballooning degeneration and the stage of NASH (Arelaki et al., 2022). The presence of NETs was also observed in the livers of STAM mice (van der Windt et al., 2018) or in another murine model (Zhao et al., 2020); iii) additional markers of neutrophils, such as myeloperoxidase (Rensen et al., 2009) and two different neutrophil serine proteases, namely neutrophil elastase (NE) and proteinase-3 (PR3) (Mirea et al., 2019), have been described in NAFLD/NASH human livers with NE and PR3 found to be associated with NASH and/or liver fibrosis; iv) mechanistic attempts to block or inhibit the function of neutrophils, for example by using mice deficient in either NE or myeloperoxidase and fed on dietary protocols to induce NASH, resulted in a significant reduction of liver injury (Pulli et al., 2015; Chen et al., 2019). Of course, the possible involvement of neutrophils relies on their ability to potentially contribute to hepatocyte injury and death by releasing proteases, ROS and other oxidants, NETs and cytokines (Jorch and Kubes, 2017; Soehnlein et al., 2017; Peiseler and Kubes, 2019). As a note of caution, the presence of neutrophils in NASH livers, particularly at the onset of the disease progression, does not mean that they are relevant as macrophages (either KCs and MoMs) and, unfortunately, we are unaware of studies employing scRNAseq or other omics specifically dedicated to neutrophils in NASH.

5.3. The role of cells of adaptive immunity

As evident from the previous sections, innate immune cells and related mechanisms play a key role in sustaining persistent inflammation in NASH patients and murine models. However, mounting evidence also indicate that adaptive immunity and related cells can afford a significant contribution to the progression towards fibrosis and cirrhosis (Sutti and Albano, 2020; Peiseler et al., 2022) as well as to the development of HCC in NASH (Cannito et al., 2023).

5.3.1. NASH is characterized by liver infiltration of B- and T-lymphocytes

The involvement of adaptive immune cells is supported by the histopathologic evidence in NASH patients of B- and T-lymphocytes infiltrated in lobular and periportal areas (Gadd et al., 2014; Yeh and Brunt, 2014). These B- and T-lymphocytes have been described to form focal aggregates (Bruzzì et al., 2018), resembling ectopic lymphoid-like structures described in other pathological conditions (Pitzalis et al., 2014), which positively correlates with lobular inflammation and the score for fibrosis (Bruzzì et al., 2018). Similar observations have been reported also for murine dietary models of NASH where infiltration of B cells and $CD4^+$ and $CD8^+$ T-lymphocytes parallels the worsening of parenchymal injury and lobular inflammation (Sutti et al., 2014; Wolf et al., 2014; Grohmann et al., 2018). B-lymphocytes involved are $CD43^-/CD23^+$ whereas the infiltration of T-lymphocytes involves proinflammatory $CD4^+$ and interferon-γ (IFN-γ)-producing T-helper 1 (Th-1) cells, IL-17 producing T-helper 17 (Th-17) cells and cytotoxic $CD8^+$ T-cells (Sutti and Albano, 2020; Ramadori et al., 2022). Infiltrating T-lymphocytes are functionally active, having an increased production of the cytokine LIGHT (also known as tumor necrosis factor superfamily 14 or TNFSF14) and express memory or effector markers like CD25, CD44 and CD69 (Sutti et al., 2014; Wolf et al., 2014; Grohmann et al., 2018). The role of adaptive immunity in NASH has been mechanistically confirmed by the evidence that steatosis, hepatocyte injury and lobular inflammation are significantly decreased in immunocompromised $Rag1^{-/-}$ mice, which are lacking mature B- and T-lymphocytes and NKT cells, likely mediated by FFAs uptake by hepatocytes stimulated by LIGHT (Wolf et al., 2014), or following selective ablation of either B- or T-lymphocytes (Barrow et al., 2021; Ramadori et al., 2022). An elegant murine study employing mice carrying hepatocyte-specific deletion of T cell protein tyrosine phosphatase (TCPTP), which is responsible for nuclear dephosphorylation of transcription factors belonging to the family of signal transducer and activator of transcription 1 (STAT), has indicated that lymphocyte recruitment in the high fat diet model of NASH depends on the stimulation of STAT1 activity that promotes the increased expression of the lymphocyte chemokine CXCL9 (Grohmann et al., 2018). Interestingly, CXCL9 levels have been reported to increase in obese patients with NAFLD vs patients without steatosis and, particularly, to be even higher in obese NASH patients (Grohmann et al., 2018). Whether $CD4^+$ T-lymphocytes are concerned, their recruitment into NASH liver has been proposed to be also related to increased expression of vascular adhesion protein 1 (VAP1), an amino-oxidase constitutively expressed on human hepatic endothelium; serum levels of the soluble form or sVAP1 are significantly higher in NASH patients than in patients with steatosis or in healthy controls (Weston et al., 2015).

5.3.2. The role of $CD4^+$ T helper cells and T_H17 cells in NAFLD/NASH

As previously mentioned, $CD4^+$ T helper cells have been detected in the liver of either murine NASH or patients (Inzaugarat et al., 2011; Sutti et al., 2014; Wolf et al., 2014; Weston et al., 2015; Grohmann et al., 2018), including pediatric ones (Ferreyra Solari et al., 2012). According to literature data, $CD4^+$ T helper cells during NASH have been reported to polarize into interferon γ (IFNγ)-producing T helper 1 (T_H1) cells (Sutti et al., 2014) positive for the Tbet transcription factor (Li et al., 2005). Accordingly, both adult and pediatric NASH patients showed increased circulating and hepatic levels of IFNγ-producing $CD4^+$ T cells (Inzaugarat et al., 2011; Ferreyra Solari et al., 2012). Accordingly, plasma levels of IFNγ correlated with the severity of fibrosis and with the number and size of lymphocyte aggregates in NASH patients (Bruzzì et al., 2018) and genetically induced deficiency of IFNγ resulted in a significant attenuation of inflammation and fibrosis in a NASH dietary murine model (Luo et al., 2013). In conditions of NASH, it has been also reported the involvement of 17 T helper (T_H17) cells that can differentiate from $CD4^+$ T helper cells in response to inflammatory conditions and mediators. Circulating and liver T_H17 cells, which are known to produce cytokines of IL-17 family (IL-17 A-F) and other cytokines (IL-21, IL-22, IFNγ, TNFα), have been reported to increase in number in NAFL as well as NASH patients (Tang et al., 2011; Molina et al., 2019), with a significant increase in hepatic T_H17 cells correlating with progression towards NASH (Rau et al., 2016). Similar results were obtained in dietary models of murine NASH and mechanistically confirmed by a significant reduction of steatohepatitis detected in mice deficient for either IL-17A and IL-17F or for the IL-17A receptor (IL-17RA) (Harley et al., 2014; Giles et al., 2016; Gomes et al., 2016) which was accompanied by an overall decrease in pro-inflammatory mediators as well as macrophage and T cell recruitment (Giles et al., 2016). Interestingly, experimental studies suggest that the contribution of T_H17 cells to lobular inflammation during NASH progression may be antagonized or

prevented by $CD4^+$ T_H22 cells and the expression levels of IL-22, since deficiency of IL-17 and IL-22 exerted opposite effect on experimental fibrosis (Rolla et al., 2016; Molina et al., 2019).

5.3.3. The role of $CD8^+$ cytotoxic T lymphocytes in NAFLD/NASH

Differently from what was just described for T_H1 and T_H17 cells, the role of $CD8^+$ cytotoxic T-lymphocytes in NAFLD/NASH is still incompletely understood, although different laboratories have documented an increased presence of these cells in the liver of either patients or experimental mice (Sutti et al., 2014; Wolf et al., 2014; Ghazarian et al., 2017; Bhattacharjee et al., 2017; Grohmann et al., 2018). Few data from murine dietary models are at present available, including the following: i) in a dietary model of NASH cytotoxic T-lymphocytes have been reported to be recruited into injured murine liver in response to IFNα-mediated signals and to promote insulin resistance and regulate gluconeogenesis (Ghazarian et al., 2017); ii) by employing genetically manipulated mice depleted of $CD8^+$ cytotoxic T-lymphocytes and NKT cells and fed on a choline-deficient high-fat diet, a protection against steatosis and steatohepatitis as well as a reducton of production of LIGHT has been reported (Wolf et al., 2014); iii) the selective ablation of $CD8^+$ cytotoxic T-lymphocytes was reported to ameliorate experimental NASH in mice fed on a high-fat, high-carbohydrate (HF–HC) diet (Bhattacharjee et al., 2017); iv) of interest, $CD8^+$ T cells were reported to directly activate HSCs *in vivo* in murine NASH liver as well as in in vitro experiments (Breuer et al., 2020). In more recent studies performed on NASH patients, the following major issues have emerged: i) when NASH patients were analysed by means of transcriptional and immune profiling, this analysis revealed a hepatic NASH-related gene signature indicating the presence of immune-associated genes linked to inflammatory responses, antigen presentation and $CD8^+$ cytotoxic T cells. Indeed, $CD8^+$ cytotoxic T cells as well as the two variants of dendritic cells (DCs) cDC1 and cDC2 cells were increased in the blood of NASH patients, a scenario that in the same study was also observed in a murine dietary model of NASH (Haas et al., 2019); ii) a recent single cell sequencing study has identified in NASH patients and in murine NASH an expanded population of "autoaggressive" $CD8^+$ cytotoxic T cells expressing markers indicative of tissue residency like CXCR6, exhaustion like programmed cell death −1 (PD-1) and of effector function like Granzyme B (Dudek et al., 2021; Barsch et al., 2022); $CXCR6^+$ $CD8^+$ T cells were peculiarly able to directly kill hepatocytes in a MHC class-I antigens independent manner and the hyperactivation of these cells was proposed to be related to metabolic stimuli and to operate mainly at an advanced stage of NASH progression (Dudek et al., 2021).

5.3.4. The role of B-lymphocytes in NAFLD/NASH

The presence of B-lymphocytes has been unequivocally detected in liver biopsies from NAFLD patients and within cell aggregates rich in T-lymphocytes (Bruzzì et al., 2018; Grohmann et al., 2018). According to data from murine models, B cells were activated in parallel with the development of NASH (Bruzzì et al., 2018) and in a T cell-independent manner to become mature plasma cells (Tsiantoulas et al., 2015). In murine NASH most B cells expressed a $B220^+IgM^+CD23^+CD43^-$ B2 phenotype resembling the one of spleen follicular B cells and only a minor fraction of $B220^+IgM^+CD23^-CD43^+$ B1 cells was observed (Novobrantseva et al., 2005). This is of interest since B2 cells require $CD4^+$ T helper cells to proliferate and to produce and highly release antigen-specific IgA, IgG or IgE, whereas B1 cells, following antigen stimulation but independently on T cells, mature into plasma cells releasing IgM natural antibodies (Tsiantoulas et al., 2015). In murine NASH, B2 cells (i.e., $CD43^-CD23^+$ cells) involvement is paralleld by increased hepatic expression of B cell - activating factor (BAFF) (Pitzalis et al., 2014), a cytokine contributing to B cell survival and maturation (Tsiantoulas et al., 2015) and recently confirmed as potentially relevant in fibrosis progression in murine NASH (Kanemitsu-Okada et al., 2023). Circulating BAFF levels, which are significantly increased in NASH patients versus patients with simple steatosis, correlates with the severity of steatohepatitis and fibrosis in humans (Miyake et al., 2013). Accordingly, a mechanistic experimental study employing genetically manipulated mice showed that the overexpression of a soluble form of the BAFF-APRIL receptor transmembrane activator and cyclophilin ligand interactor (TACI) prevented maturation into plasma cells and resulted in attenuated inflammation and fibrosis (Bruzzì et al., 2018). Moreover, still in murine mice B cells developed into plasma cells producing IgG directed versus oxidative stress – derived epitopes (anti-OSE) (Bruzzì et al., 2018), a stimulating data since NASH patients were previously reported to exhibit elevated plasma anti-OSE antibody titers (Albano et al., 2005).

From a functional point of view, B lymphocytes contribution to NASH progression may be envisaged as the consequence of the production of pro-inflammatory mediators and of their antigen presenting ability (Lund, 2008; Di Lillo et al., 2011). Interestingly, at least in experimental NASH, the activation of B cells seems to precede the recruitment of either $CD4^+$ or $CD8^+$ T-lymphocytes whereas to interfere with B2 cells results in a reduction of TH1 cell activation and a decrease of IFNγ release by $CD4^+$ T cells (Bruzzì et al., 2018). Moreover, it has been suggested that B cells, through the release of pro-inflammatory mediators, may stimulate both liver macrophages and HSCs, with activated HSCs being able to sustain B cell survival and their maturation into plasma cells through the release of retinoic acid (Thapa et al., 2015). Few related data are available for NASH patients and one study proposed that serum levels of IgA (which are produced by B2 cells), that were markedly increased in NASH patients as compared to patients with fatty liver, may represent an independent predictor of advanced liver fibrosis (McPherson et al., 2014; Shalapour et al., 2017). Indeed, in a murine model of NASH the deficiency of IgA reduced steatohepatitis (Shalapour et al., 2017). The ability of B cells to interact with HSCs in a pro-fibrogenic manner has been confirmed more recently in a study in which sc-RNAseq and ligand-receptor network analysis of NASH livers were performed, showing that activated HSCs express increased CXCL12 with CXCR4 in B cells as the potential target (Xiong et al., 2019). Moreover, clusters of mature and plasma B cells were detected by scRNAseq in areas of intense fibrosis in cirrhotic patients (Ramachandran et al., 2019). Finally, a very recent study has offered additional evidence for a role of B cells in sustaining NASH-related fibrosis focusing this time the attention on intestinal B cells. Mechanistically, genetic or pharmacological depletion of these cells, that were found increased in both murine and human NASH samples, resulted in the prevention or reversion of experimental NASH and liver fibrosis. Authors suggest that B cells may have a dual role in NASH pathogenesis, being implicated in the activation of auto-aggressive T cells and the development of fibrosis via activation of monocyte-derived macrophages by secreted immunoglobulins (e.g., IgA) (Kotsiliti et al., 2023).

5.4. The interactions between innate and adaptive immunity in NAFLD: of DCs, NKT, Tregs and mechanisms promoting adaptive immunity

Available literature evidence indicates that in NASH liver there is an overall alteration of those mechanisms that physiologically contribute to immune-suppression and tolerance induction versus autoantigens and antigens arriving from the gut (i.e., from food or bacteria) (Crispe, 2014; Horst et al., 2016). These altered mechanisms, in response to several intra- and extrahepatic stimuli, can subvert or significantly modify the already very complex scenario mediated by stimuli involving the interactions between professional antigen-presenting cells (APCs, like KCs and dendritic cells or DCs), unconventional APCs (hepatocytes, HSCs, sinusoidal endothelial cells), T lymphocytes as well as NK and NKT cells (Sutti and Albano, 2020). The interplay between innate and adaptive immune cells is an established issue since cytokines released by T_H1, T_H17 and $CD8^+$ T lymphocytes represent very effective stimuli for the activation of macrophages that, in turn, can release a number of cytokines and chemokines (IL-12, IL-23, CXCL9, CXCL10, CXCL11) able to affect functions of lymphocytes (Krenkel and Tacke, 2017). In addition,

the release of IL-15 and IL-18 by macrophages has been reported to promote NK activation that have a role in NASH and fibrogenesis (Tosello-Trampont, et al., 2017). Along these lines, a very recent study investigated the differences between peripheral blood immune cells in patients with different stages of NAFLD by using cytometry by time of flight (CyTOF) and related bioinformatics analysis. This study detected changes in peripheral immune cells in early and late stages of NAFLD describing a scenario in which innate and adaptive immune cells like B- and T lymphocytes, NK cells and monocytes changed numerically in peripheral blood and become less active (Waller et al., 2023).

5.4.1. The role of denditic cells in NASH

Liver DCs have a relevant role in orchestrating liver immunity and modulating liver fibrogenesis (Rahman and Aloman, 2013), although they are generally considered as less efficient then DCs in other tissues in mediating interactions with T lymphocytes (Jenne and Kubes, 2013). Their role in the pathogenesis of NASH is still incompletely understood but some recent studies suggest that subsets of DCs, may promote inflammatory response. Different laboratories have reported an accumulation of DCs in the liver of NASH patients (Henning et al., 2013; Haas et al., 2019). The use of single cell transcriptomic analysis allowed to identify the predominant subset of DCs in human NASH livers as CXCR1-expressing conventional DC type 1 (or cDC1) cells, one of the two classical DC subset (Deczkowska et al., 2021). Although also cDC2 cells were increased, only the number of cDC1s cells was found to correlate with the severity of NASH (Deczkowska et al., 2021). In the same study other very interesting findings were reported: i) the conditions of NASH-were able to induce an increase of cDCs progenitors in bone marrow as well as in peripheral blood; ii) sequencing of physically interacting cDC-T cell pairs from liver lymph nodes revealed that cDCs in NASH can promote inflammatory T cell reprogramming and, likely, worsen NASH; iii) the use of mice genetically manipulated to deplete cDC1 cells resulted in attenuation of experimental NASH (Deczkowska et al., 2021). Moreover, as recently reviewed (Brombacher and Everts, 2020), the metabolic microenvironment can deeply influence the shaping of DCs and their function. It has been reported in both mice and patients that the lipid content in hepatic DCs may have a relevant role since DCs containing high levels of lipids were reported to be immunogenic, rather than to mediate tolerance, by activating T lymphocytes as well as NK and NKT cells. The immunogenicity of lipid-rich liver DCs required the secretion of TNFα whereas DCs containing low levels of lipids were less immunogenic (Ibrahim et al., 2012).

5.4.2. The role of NKT cells in NAFLD/NASH

NKT liver cells represent a subset of T cells characterized by the expression of T cell receptor and NK cell surface receptors, like CD56 or CD161 in humans and NK1.1 in mice, (Marrero et al., 2018b). Liver NKT cells can recognize lipid antigens shown by APCs and can release a number of cytokines able to activate T_H1, T_H2 as well as $CD4^+CD25^+$ regulatory T (Treg) cells, including IL-4, IL-10, IFNγ and TNFα, with the majority of liver NKT cells belonging to type I or invariant NKT cells (iNKT) (Marrero et al., 2018b). NKT activity can either stimulate or suppress inflammatory and immune response (Sutti and Albano, 2020) and it has been hypothesized that the presence of NKT may vary during NAFLD progression: i) NKT are decreased in the presence of simple steatosis and in the early phase of NASH, possibly in relation to the expression of IL-12 and/or T cell mucin domain-3/galectin-9 (Kremer et al., 2010; Tang et al., 2013); ii) NKT cells are clearly expanded in number in a more advanced stage of the disease in relation to the release of cytokines like IL-17A, IFNγ and LIGHT (Syn et al., 2012; Wolf et al., 2014). According to the issues of a late involvement of NKT in NASH, experimental studies designed to interfere with NKT cells during advanced NASH have significantly attenuated liver injury, inflammatory response and fibrosis (Syn et al., 2012; Wolf et al., 2014; Bhattacharjee et al., 2017; Maricic et al., 2018). Moreover, the expansion of liver NKT cells in advanced NASH has been reported to depend on CXCL16-dependent increased recruitment and on IL-15 mediated differentiation or survival (Gordy et al., 2011; Wehr et al., 2013; Sutti et al., 2014). Interestingly, and remarking the possible critical role of IL-15, mice lacking IL-15 or the cognate receptor α (IL- 15Rα) had a decreased infiltration of NKT cells as well as of $CD4^+$ and $CD8^+$ cells; moreover, these mice exhibited a reduction of steatosis and lobular inflammation when fed on a high fat diet as compared to wild type mice (Cepero-Donates et al., 2016). Finally, a study performed on $Ja^{-/-}$ mice, which lack iNKT cells, also suggested a strict relationship between iNKT cells and $CD8^+$ cytotoxic T lymphocytes (Maricic et al., 2018).

5.4.3. The role of NK cells in NAFLD NASH

NK cells have been originally reported to be able to selectively target and kill activated HSCs, possibly by releasing IFNγ (Melhem et al., 2006; Radaeva et al., 2006; Gur et al., 2012). However, it was early suggested that HSCs in advanced fibrosis were able to inhibit NK cells through the release of TGFβ (Jeong et al., 2011). Recently, in a murine model of NASH an increased TGFβ signaling led to the loss of NK cell cytolytic activity, an event interpretated as an adaptive mechanism to reduce liver inflammation (Cuff et al., 2019) Although the recruitment of NK cells seem to be increased in NASH liver, such a recruitment has been correlated with a more severe disease (Martinez-Chantar et al., 2021). Along these lines, NK cells isolated from a cohoort of NAFLD/NASH patients were not able to kill HSCs when obtained from patients having severe insulin resistance and an advanced stage of fibrosis (Amer et al., 2018).

5.4.4. The role of regulatory T cells (Treg) in NAFLD NASH

A critical role in the regulation of immune tolerance in liver parenchyma is exerted by Treg cells, a subset of $CD4^+$ T cells expressing the transcription factor Forkhead boxprotein 3 (FOXP3). Liver DCs are believed to have a role in driving $CD4^+$ T cells differentiation towards Treg by expressing membrane-bound programmed cell death 1 ligand 1 (PD-L1) and by releasing IL-10 and kynurenine (Crispe, 2014). Treg cells are able to suppress, through the expression of co-inhibitory molecule cytotoxic T lymphocyte antigen-4 (CTLA-4) as well as the release of IL-10 and TGFβ, proliferation and function of both $CD4^+$ and $CD8^+$ T lymphocytes (Crispe, 2014). Concerning progressive NAFLD it has been reported that patients with steatosis have a reduced number of resting Treg cells in peripheral blood versus healthy humans and that the number of circulating resting Treg is even lower in NASH patients. This reduction has been found to parallel the expansion of T_H1 and T_H17 cells. By contrast, although in NAFLD patients the number of circulating activated Treg is increased, no significant change in the number of hepatic Treg has been reported; in the same study Authors proposed that the ratio between liver levels of T_H17 cells and resting Treg may help to distinguish NASH patients from patients with simple steatosis (Rau et al., 2016). It is interesting to note that in obese patients a closely similar reduction of Treg and an alteration of the ratio between T_H17 cells and Treg cells has been detected in visceral adipose tissue (McLaughlin et al., 2017). At present the precise mechanism(s) leading to a reduction of Treg cells in NAFLD is a matter of speculation and several hypotheses have been raised (Sutti and Albano, 2020). Moreover, it should be noted that conflicting results concerning the relevance of the role of Treg have been obtained in murine models which have reported either no difference in the number of Treg in experimental NASH livers (Rolla et al., 2016; Grohmann et al., 2018) or, by depleting Treg cells, a worsening of steatohepatitis (Chatzigeorgiou et al., 2014).

5.4.5. The emerging role of MAIT cells and γδ T cells in NASH

Mucosal-associated invariant T (MAIT) cells and γδ T cells are the most relevant cellular components of so-called *unconventional T cells*, which represent approx. 10% of circulating T cells. However, these cells represent most T cells in the liver (Pellicci et al., 2020) and for this reason they have been recently investigated in relation to the pathogenesis of NASH. Whether MAIT cells are concerned, one should note

that these cells in humans represent approx. 15–45% of liver T cells, with invariant NKT (iNKT) cells representing no more than 1% of T cells. This scenario is completely reversed in mice liver where MAIT cells are relatively rare whereas iNKT represent 30–50% of liver T cells (Heymann and Tacke, 2016; Peiseler et al., 2022). Data on the role of MAIT cells in CLD (see also Czaja, 2021) emerged starting from 2018 in several clinical studies that indicated that MAIT cells in patients with NASH were increased in fibrotic septa (Hegde et al., 2018) as well as their overall number in NASH livers (Li et al., 2018c). However, MAIT cells decreased in peripheral blood where these cells were functionally altered, as indicated by the increased expression of IL-4 and CXCR6 but reduced expression of TNFα and IFNγ (Li et al., 2018c). In the same year a third study, this time investigating the role of activated MAIT cells in auto-immune liver diseases (AILD), first proposed that they can exert a direct pro-fibrogenic role since MAIT cells obtained from both healthy and AILD patients were reported to express an exhausted phenotype but, of interest, able to induce an activated, proinflammatory and profibrogenic phenotype in hHSCs in vitro that was partly mediated by IL-17 (Bottcher et al., 2018). Additional but sometimes contradictory data have been provided by murine studies that can be summarized as follows: i) in a dietary murine model of NASH the depletion of MAIT cells resulted in a worsening of liver injury, suggesting a protective role for these cells, possibly by means of inducing polarization of macrophage to an anti-inflammatory phenotype (Li et al., 2018c); ii) similarly, a very recent study reported that inhibition of MAIT cells promoted fibrosis regression by reprogramming macrophage phenotype from Ly6C^{high} to the restorative Ly6C^{low} one (Mabire et al., 2023); ii) by contrast, another study reported that in obesity MAIT cells may exert a pro-inflammatory role in adipose tissue, likely by polarizing macrophages to a pro-inflammatory phenotype, but also by promoting gut dysbiosis and metabolic dysfunction (Toubal et al., 2020); iii) in a more recent study Vα19 mice, in which the number of MAIT cells is equivalent to- or greater than that in humans, were fed on a high fat diet; it was found that Vα19 mice exhibited a reduction of steatosis, NAFLD activity score, and of transcripts relevant to lipogenesis as compared to control mice but without significant changes in glucose tolerance, insulin sensitivity, inflammation in adipose tissues, or intestinal dysbiosis (Kishi et al., 2022).

Concerning hepatic γδ T cells, the roles and functions of these cells in progressive NAFLD are still just partially understood and few data are available at present. These cells are a subset of CD3$^+$ T cells characterized by a T cell receptor (TCR) formed by γ and δ chains that do not require MHC-mediated antigen presentation and can release IL-17A. In a dietary murine model of NASH, γδ T cells were found to be increased in liver and adipose tissue and to be related to disease progression through the release of IL-17A, as confirmed by attenuation of NASH in mice following selective depletion of the cytokine in γδ T cells (Li et al., 2017). The recruitment of γδ T cells into injured liver has been reported to depend on CCL20 and its receptor CCR6 (Haas et al., 2009). Moreover, mice made deficient for CCR6 showed a worsening of inflammation and fibrosis when fed on different dietary models of NASH, a feature that was reverted by adoptive transfer of γδ T cells and proposed to be related to induction of apoptosis in HSCs (Hammerich et al., 2014). This attenuation of NASH was observed also in CD1d-deficient mice (Syn et al., 2012), a data potentially of interest since in both mice and humans the existence of CD1d restricted γδ T cells recognizing lipid antigens has been reported (Pellicci et al., 2020). An increased expression of the mentioned ligand/receptor pair CCL20/CCR6 was found to be increased also in human cirrhosis (Hammerich et al., 2014). Moreover, in the liver of human NAFLD patients Vδ2$^-$ γδT cells were detected at higher frequencies whereas Vδ2$^+$ γδT cells were detected at slightly lower frequencies as compared to healthy controls (Diedrich et al., 2020).

5.4.6. A possible role for extrahepatic factors in down-modulating liver immunotolerance

As previously suggested, in conditions of NASH the livers might lose their immune tolerance following the impact of either intrahepatic or extrahepatic factors. If extrahepatic factors are concerned, evidence coming mainly from pre-clinical studies with dietary models of NASH indicate a number of factors that may contribute to loss of liver immune tolerance. NASH-related conditions of chronic inflammation can prevent or even abolish the role of KCs in promoting the expansion of Treg cells and then of tolerogenic responses, favouring the activation of CD4$^+$ T lymphocytes (Heymann et al., 2015). Similarly, liver immune tolerance may be dampened by DAMPs as well as other factors released by injured hepatocyte, including oxidative stress – related epitopes, that presented by APCs may directly activate B- and T-lymphocytes (Sutti and Albano, 2020). A major contribution is likely to be offered by gut dysbiosis, described in patients with NAFLD and NASH (although not in all patients) and related evidence can be synthetized as follows: i) an excess presence of LPS or other endotoxins in the portal blood may shift the usual anti-inflammatory response of KCs and sinusoidal endothelial cells (SECs) through IL-10 release into a more deleterious (i.e. for disease progression) pro-inflammatory one which involves mainly KCs (Henao-Mejia et al., 2012; Schneider et al., 2015); ii) liver CD8$^+$ T cells can be directly activated to release IFN type I and accumulated in the liver by PAMPs (i.e., bacterial products) (Ghazarian et al., 2017); iii) alteration of gut tolerance to autoantigens may have a role (Li et al., 2018a) and it has been related to the action of SCFAs (like propionate, butyrate and acetate produced by bacteria-mediated fermentation) detected in NASH patients (Weismann and Binder, 2012; Bashiardes et al., 2016; Eckert et al., 2016); increased levels of SCFAs have been reported in association with increased numbers of T_H17 cells and reduced number of Treg cells in preripheral blood of NASH patients (Rau et al., 2018).

6. Myofibroblasts, pro-fibrogenic mechanisms and cell interactions in progressive NAFLD

6.1. General concepts on liver MFs: origin and phenotypic responses

Liver MFs represent an heterogenous population of α-smooth muscle actin (αSMA) positive cells, known to play a major pro-fibrogenic role in CLDs, that can originate from different precursor cells of mesenchymal origin through a process defined as of activation/transdifferentiation (Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Koyama and Brenner, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Friedman and Pinzani, 2022). The fully activated pro-fibrogenic MF phenotype in chronically injured liver is a rather unique example of a cell type able to receive, integrate and respond to the multiple signals and cell-to-cell interactions occurring in the fibrogenic microenvironment. These signals include ROS (from damaged hepatocytes or activated innate immune cells) as well as a plethora or growth factors, cytokines, chemokines, adipokines, pro-angiogenic factors and several others released by hepatic and extrahepatic cells involved in CLD progression and mentioned in the previous sections (Tsuchida and Friedman, 2017). In addition, in the specific conditions of progressive NAFLD these cells can also sense and respond to metabolic signals as well as signals related to gut dysbiosis or delivered from adipose tissue (Trivedi et al., 2021). According to current literature, liver MFs in CLDs, then also in NAFLD/NASH, mainly originate from activation/transdifferentiation of HSCs, sometimes referred to as HSC/MFs (Friedman, 2008; Tsuchida and Friedman, 2017), as also suggested by fate tracing studies (Mederacke et al., 2013). However, it should be briefly recalled that a significant number of MFs can also originate from activation/transdifferentiation of portal fibroblasts, particularly in conditions of chronic injury that target biliary epithelium (i.e., biliary fibrosis like in PBC and PSC as well as in opther cholangiopathies), but also detected in CLDs of different aetiology (Dranoff and Wells, 2010; Wells and Schwabe, 2015). In addition, a limited percentage of cells has been described to originate following liver recruitment and consequent activation/transdifferentiation of precursor cells derived from bone

marrow (mesenchymal stem cells, fibrocytes), as shown in both clinical and experimental studies (Forbes et al., 2004; Russo et al., 2006; di Bonzo et al., 2008). A minor numerical contribution to liver MFs may be offered by mesothelial cells, possibly through a mesothelial-to-mesenchymal transition (Luo et al., 2013) whereas the contribution of epithelial-to-mesenchymal transition to liver fibrogenesis is at present controversial and, likely, of minor relevance (Forbes and Parola, 2011; Parola and Pinzani, 2019).

Whatever the different origin, fully activated liver MFs operate a number of common phenotypic responses, mostly characterized in studies related to HSC/MFs (Friedman, 2008; Tsuchida and Friedman, 2017; Trivedi et al., 2021) that can be briefly summarized as follows: i) following injury, liver MFs are highly proliferative cells that mainly respond to PDGF but also to other mitogens like TGFα, thrombin, CTGF, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leptin; ii) most of mitogens, plus TGFβ1, are likely to be responsible for MFs survival and resistance to the induction of apoptosis; iii) liver MFs are considered the most relevant cell type for increased deposition of ECM components, mainly fibrillar collagen type I and type III; moreover, under conditions of chronic injury MFs are reprogrammed to overexpress tissue inhibitor of metalloproteases (TIMPs) as well as metalloproteases (MMPs) not or less efficient to remove fibrillar collagens and the excess of ECM components; this happens mainly in response to TGFβ1 but also as a response to other growth factors (CTGF, bFGF) as well as ROS and related intermediates; iv) liver MFs have a role in inflammatory response since they express receptors for many cytokines, chemokines and other mediators but, following activation, can also contribute by releasing chemoattractants like CCL2 and CCL21 as well as IL-1β; as it will be mentioned in the next section, they can modulate behaviour and function of both innate and adaptive immune cells; v) liver MFs can migrate in response to several chemoattractant polypeptides including PDGF, CCL2, VEG, angiotensin I and Angiopoietin I; with most of these polypeptides the migration of MFs involves intracellular generation of ROS following ligand-receptor – mediated activation of NADPH-oxidase and activation of ERK and JNK pathways (Novo et al., 2011) or under hypoxic conditions (Novo et al., 2012); vi) liver MFs, which are also sensitive to hypoxic conditions, can release pro-angiogenic mediators like VEGF, Angiopoietin-1 or -2, hedgehog ligands or PDGF, then contributing to neoangiogenesis that parallels fibrogenesis and the formation of fibrotic sepata (Bocca et al., 2015; Lemoinne et al., 2016); along these lines one should recall that hypoxia and hypoxia-inducible factors have an emerging role in sustaining fibrogenic CLD progression, including NASH (Morello et al., 2018; Foglia et al., 2021); vii) liver MFs can also contract and relax in response to a variety of vasoactive compounds (mainly endothelins and NO).

6.2. *HSCs heterogeneity: old and novel paradigm in the single cell transcriptome era*

HSCs are cells derived from the septum transversum mesenchyme during embryo development (Asahina et al., 2009, 2011) than in adult liver reside in the space of Disse, at strict contact with hepatocytes and SECs, where they are able to produce and remodel the ECM components (Friedman, 2008; Tsuchida and Friedman, 2017). Moreover, physiologically HSCs have a well characterized role in storing vitamin A as retinyl esters and a role as liver specific pericytes (Blomhoff and Wake, 1991; Pinzani et al., 1992). When activated, HSCs loose droplets containing vitamin A and retinoids to assume all the typical characters and phenotypic responses of MFs and as recently reviewed, have a role in regulating critical processes such as hepatic growth, inflammation and immune responses but also energy and nutrient homeostasis (Trivedi et al., 2021). According to the fact that HSCs are widely recognized as the major source of liver MFs in CLDs, most of available data refer to HSCs and then to HSC/MFs. Established literature data indicate that HSCs, under conditions of chronic liver injury, can undergo the mentioned process of activation/transdifferentiation leading so called "*quiescent*" HSCs to switch on the "*activated*" and MF-like phenotype previously described that has a major role in the fibrogenic progression of a CLD (Tsuchida and Friedman, 2017). Along these lines, when the condition or agent leading to liver injury is removed, HSC/MFs decline in number either by means of apoptosis or through a reversion to an "*inactive*" phenotype which express a peculiar epigenetic signature and have been reported to reactivate rapidly in case of re-injury (Kisseleva et al., 2012; Troeger et al., 2012; Liu et al., 2020). The activation of HSCs, not differently from what previously anticipated for MFs, is known to be triggered by the plethora of signals already mentioned in the previous section, with HSCs responding by eliciting pro-fibrogenic paracrine and autocrine loops that include at least TGFβ1, PDGF, VEGF, CCL2 and possibly CTGF (Higashi et al., 2017).

However, the use of single-cell transcriptomic methods has recently revealed that HSCs represent a more heterogenous population of what previously believed (Dobie et al., 2019; Krenkel et al., 2019; Ramachandran et al., 2019, 2020; Xiong et al., 2019). A first set of data specifically concerning HSCs has emerged from a scRNAseq study designed to investigate these cells in healthy and fibrotic liver by using Pdgfrb-GFP knockin reporter mice (a model allowing to label all mesenchymal cells in the mouse liver) and a murine model of centrilobular necrosis (Dobie et al., 2019). This study revealed spatial zonation of HSCs across the hepatic lobule and, by identifying specific gene signatures and markers, also revealed that HSCs could be partitioned into two topographic regions defined portal vein-associated HSCs (PaHSCs) and central vein-associated HSCs (CaHSCs). By using CCl_4 chronic injury as a model of pericentral injury, CaHSCs were indicated as the dominant pathogenic collagen-producing cells; interestingly, although PaHSCs also responded to centrilobular injury by increased proliferation, they did not transdifferentiate into collagen-producing MFs (Dobie et al., 2019). In a similar scRNAseq study and using the same experimental model of liver injury other Authors showed that activated HSCs may be also discriminated into three distinct subpopulations that may be indicated as i) pro-regenerative HSCs, on the basis of their elevated expression of growth factors, ii) anti-regenerative or pro-fibrogenic and iii) a mixed HSCs subpopulation (Krenkel et al., 2019), somewhat adding new concepts to the old paradigm of quiescent HSCs vs activated or inactivated HSCs.

The emerging scenario has at the same time unequivocally confirmed and significantly expanded established knowledge. Moreover, in a recent and very stimulating review it has been proposed that to support their function in healthy and injured liver HSCs should be envisaged as cells able to engage different pathways contributing to regulate carbohydrate, mitochondrial and lipid homeostasis, in addition to the known role in storing and metabolizing retinoids (Trivedi et al., 2021). These issues are somewhat out of the scope of the present review but in their analysis Trivedi et al. (2021) introduced the concept that HSCs, in order to undergo activation/transdifferentiation from quiescent to fully activated MF-like cells, need to adapt rapidly from a metabolic point of view by reprogramming carbon metabolism, by enhancing mitochondrial number and activity, ER stress as well as releasing FFAs through autophagy-dependent hydrolysis of retinyl esters in cytoplasmic droplets.

6.3. *The cross talk between HSCs, HSC/MFs and other cells in progressive NAFLD*

The idea that HSCs, both quiescent and activated and MF-like, are able to interact with other cell populations during an ongoing CLD, either as cells able to sense a variety signals/mediators as well as cells able to respond by releasing, in a autocrine/paracrine manner, is an established issue and it has been extensively reviewed in the past (Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Koyama and Brenner, 2017; Tsuchida and Friedman, 2017; Parola and Pinzani, 2019; Schwabe et al., 2020; Friedman and Pinzani, 2022). A scheme of these interactions is provided in Fig. 2. The use of single cell

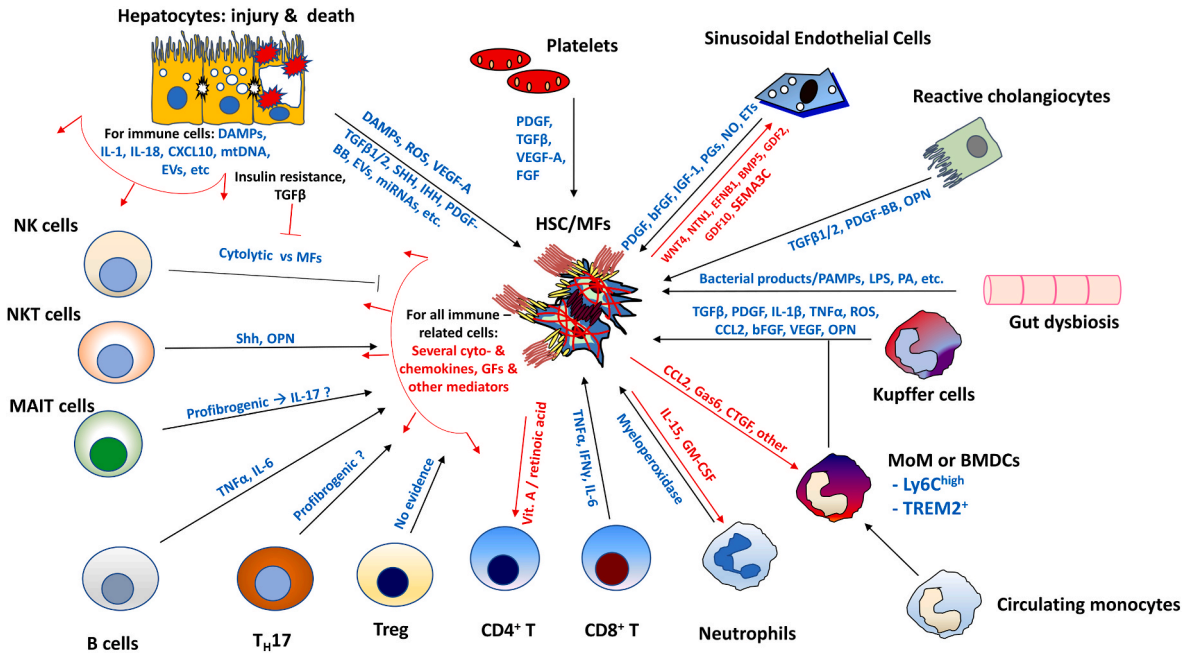

Fig. 2. Activated, MF-like, hepatic stellate cells and their cell-to-cell interactions in the hepatic scenario of NASH. Activated and MF-like hepatic stellate cells (HSC/MFs) in the NASH-related scenario of chronic liver injury can establish reciprocal interactions with practically all the other cell populations involved, including cells of epithelial origin (fat laden hepatocytes, injured and/or dying, reactive cholangiocytes), activated platelets and sinusoidal endothelial cells as well as several subsets of innate and adaptive immune cells. HSC/MFs receive signals that can sustain the MF-like phenotype and amplify pro-fibrogenic inflammatory responses and release several mediators able to modulate the response of involved cells.

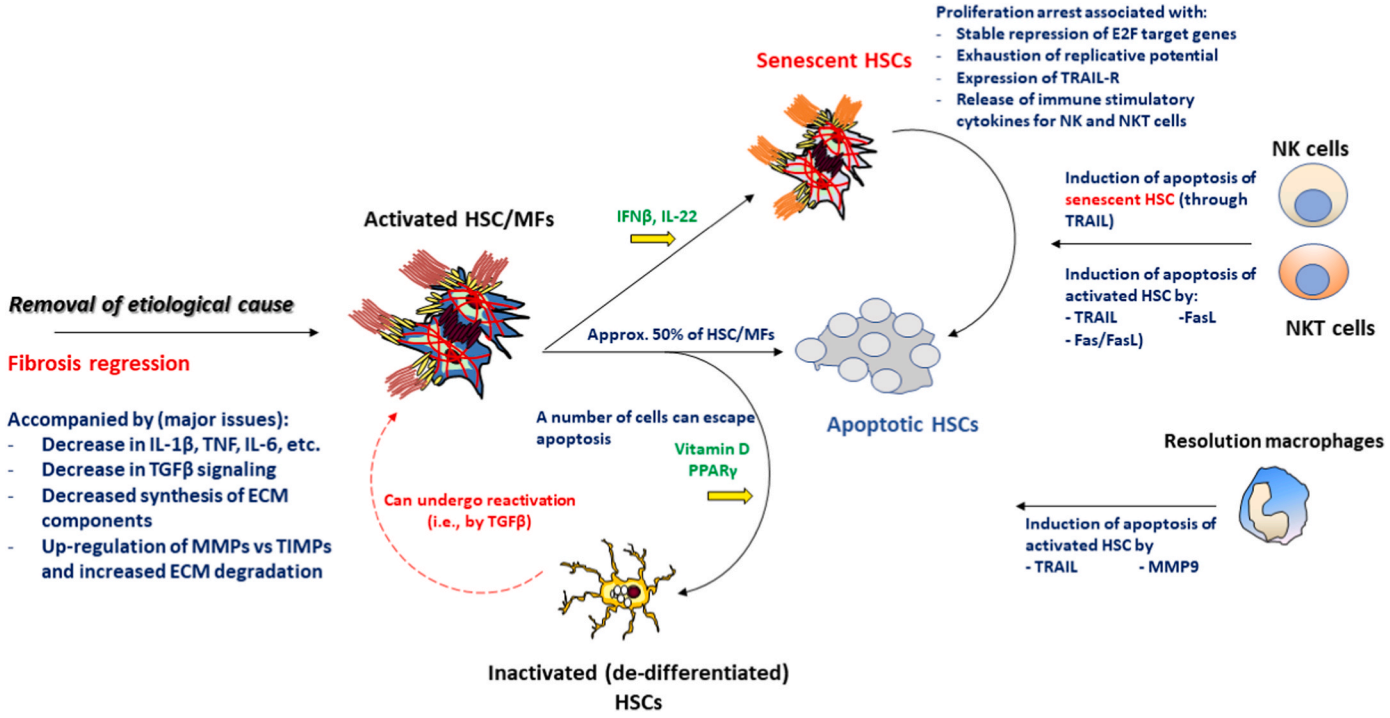

Fig. 3. Major events, cells and mechanisms involved in fibrosis regression, with a focus on phenotypic changes occurring in activated HSC/MFs. The removal of the etiological cause of CLD is the predominant event allowing fibrosis regression to occur which is accompanied by a number of relevant issues including the decrease in pro-inflammatory and pro-fibrogenic mediators, the decrease of synthesis of ECM components and the predominance of MMPs action vs those of TIMPs. Upon cessation of fibrogenic stimuli approx. 50% of HSC/MFs can undergo apoptosis, with NKT and resolution macrophages reported to contribute to the induction of apoptosis in HSC/MFs. However, some cells have been described to escape apoptosis to become inactivated or de-differentiated as well as to be even reactivated in the presence of recurrence of chronic injury and fibrogenic stimuli. Finally, HSC/MFs can also become senescent and, in turn, undergo apoptosis, possibly with the active contribution of activated NK cells.

transcriptomics and other advanced technologies has definitively provided a more precise definition and characterization of these cell-to-cell interactions in both healthy and chronically injured liver (Dobie et al., 2019; Krenkel et al., 2019; Ramachandran et al., 2019, 2020; Xiong et al., 2019).

Apart from what already reported in previous sections, here it seems worth mentioning at least a recent scRNAseq study that was specifically dedicated to investigating healthy and NASH murine liver. This elegant study offered a realistic map of the liver scenario of intrahepatic ligand/receptor signaling networks (Xiong et al., 2019). In this study Authors identified at least three well defined clusters of cells, including a HSCs cluster, an endothelial cluster and a macrophage one as prominent for paracrine and autocrine signaling. Apart from the already mentioned characterization of NAMs and of the related signature, the single cell transcriptome analysis of the HSCs cluster identified a prominent secretome including 27 genes encoding for membrane proteins and other 99 genes encoding for secreted factors. The HSCs-related protein secretome included of course structural ECM components (mainly collagens and proteoglycans) and those involved in ECM remodelling. As expected, the expression of these genes was strongly up-regulated in cells from NASH livers vs control ones. In addition, HSCs were reported to secrete 21 "*stellakines*", as for Authors' own definition, then cytokines and chemokines predicted, on the basis of the known expression of cognate receptors, to act primarily on SECs (for example WNT4, NTN1, EFNB1, BMP5, GDF2, GDF10, and SEMA3C) and immune cells, including macrophages, DCs and B- and T-lymphocytes (like the chemokines CCL2, CCL11, CXCL10, CXCL12, CXCL16 as well as CTGF and GAS6). It is interesting to note that the expression of most of these ligands released by HSCs was markedly up-regulated in NASH liver vs controls, as confirmed by a whole-liver RNA-seq analysis.

Whether the expression by activated HSCs of membrane receptors is concerned, the analysis reported an altered expression in cells obtained from NASH livers of three different categories of receptors: i) receptors implicated in liver biology and fibrosis, including PDGFRβ, FGFR2, discoidin receptor 2 (Ddr2), receptor-like Tyr kinase (Ryk), and low-density lipoprotein receptor-related protein 1 (Lrp1); ii) receptors for cytokine signaling; iii) a large variety of receptors allowing HSCs to respond to vasoactive mediators (Xiong et al., 2019). The list of expressed receptors that may promote HSCs contraction include endothelin receptor type a (Ednra), Ednrb, Angiotensin II receptor type 1a (Agtr1a), and Adrenergic receptor a 2b (Adra2b). Concerning receptors potentially inducing relaxation included transcripts for GPCRs targeted by peptide hormones such as Ramp1, Calcrl, Pth1r, and Vipr1. The expression of some of these receptors (i.e., Ednrb, Adra2b, Vipr1, Pth1r, and Ramp1), was also enriched in human HSCs.

Apart from the study by Xiong et al., other studies have outlined additional crosstalk between HSCs and non-parenchymal cells in relation to NASH. Here a few pertinent examples, not described in previous sections, can be recalled: i) a cross talk between HSCs and macrophages has been related to the expression by macrophages of the MerTK receptor that can bind different ligands, including Gas6 which is a "*stellakine*" released by HSCs; this receptor, that when activated can induce TGFβ1 expression, may be cleaved by ADAM17 to control the inflammatory response of macrophages during steatosis; however, this control does not operate in NASH and it has been suggested that this may be the consequence of a reduced availability of vitamin A (depleted in activated HSCs) necessary to activate ADAM17 (Cai et al., 2020). Moreover, a recent study has provided direct evidence that MerTK activation in macrophages can modify their secretome to promote profibrogenic features in HSCs (Pastore et al., 2022), relevant since MERTK rs4374383 polymorphism can affect the severity of fibrosis in NAFLD (Petta et al., 2016). ii) in a previous section it was mentioned that during progressive NAFLD KCs can die and that are progressively replaced by a KC-like population derived from recruited Ly-6C^{high} MoM cells; it has been proposed that HSCs, along with SECs and hepatocytes, may provide a liver niche able to recruit MoMs and to drive their differentiation into KCs (Bonnardel et al., 2019); iii) whether the interaction of HSCs with neutrophils is concerned, it is interesting to note that myeloperoxidase has been suggested to stimulate directly in culture HSCs and to activate latent TGFβ (Pulli et al., 2015); moreover, HSCs may extend the life of neutrophils through a positive feedback loop dependent of release of granulocyte-monocyte colony stimulating factor (GM-CSF) and IL-15 (Zhou et al., 2018); iv) in relation to putative interactions with NKT cells, an issue still poorly investigated, it has been shown that NKT cells obtained from a dietary model of NASH can directly activate HSCs in a co-culture system more efficiently than CD8^{+} T cells (Wolf et al., 2014).

7. The diagnosis of NAFLD/NASH and the clinical evaluation of disease progression

The most common scenario requiring a clear distinction between simple steatosis and NASH is the presence of abnormally elevated liver enzymes in an overweight/obese patient with features of metabolic syndrome with or without overt type 2 diabetes (T2D). Most commonly, this clinical situation is detected in the primary care setting and requires a robust pathway for risk stratification, with the required subsequent referrals (Tsochatzis and Newsome, 2018).

7.1. NAFLD assessment in the primary care setting

In reason of the high incidence of fatty liver in the general population, the role of primary care physicians is fundamental for prevention, screening, and care of NAFLD. In addition, early detection and management of comorbidities often associated with the presence of fatty liver leads to an overall reduction of cardiovascular and liver-related mortality (Lazarus et al., 2021).

In general terms, the diagnosis of NAFLD is based on the detection of hepatic steatosis (usually by liver ultrasonography) and exclusion of other liver diseases, particularly excessive alcohol consumption, and secondary causes of fatty liver (e.g., use of systemic steroids, amiodarone, and tamoxifen). A diagnostic flowchart based on the most followed guidelines is illustrated in Fig. 4. Once evidence of NAFLD has been confirmed, the next step is directed at verifying the possibility of NASH and liver fibrosis. It is important to stress that serum alanine aminotransferase (ALT) level correlates poorly with the presence of NASH and fibrosis (Wong et al., 2009). Because fibrosis is the histological feature with the strongest correlation with liver-related outcomes, it is sensible to focus on fibrosis assessment in patients with NAFLD (Taylor et al., 2020). Accordingly, the so-called "simple fibrosis scores" such as the Fibrosis-4 index (FIB-4) and the NAFLD fibrosis score (NFS), which are based on routine biochemical and clinical parameters, have been suggested for the use in the primary care setting. Both tests have negative predictive values of over 90% to exclude advanced fibrosis (Stages 3–4 fibrosis by the NASH Clinical Research Network System) in primary care settings (Mahady et al., 2017).

An abnormally elevated FIB-4 and/or NFS should prompt the referral to specialist care for further assessment of liver tissue fibrosis and the relative stage. In the United Kingdom, it has become established practice to perform FIB-4 followed by the ELF test, a proprietary algorytm based on ECM components, especially if the first test was indeterminate. This sequential association has increased the detection of advanced fibrosis by fivefold while reducing the number of inappropriate referrals to specialist care (Srivastava et al., 2019).

7.2. NAFLD assessment in the specialist care setting

Once a patient is referred to a specialized Hepatology service, the first requirement is to confirm the presence of liver fibrosis before proceeding to a histopathological assessment by liver biopsy. Elastography is the most validated system to quantify liver fibrosis by measuring the propagation velocity of ultrasound waves passing through the liver parenchyma. This considering that as fibrosis progresses, liver tissue

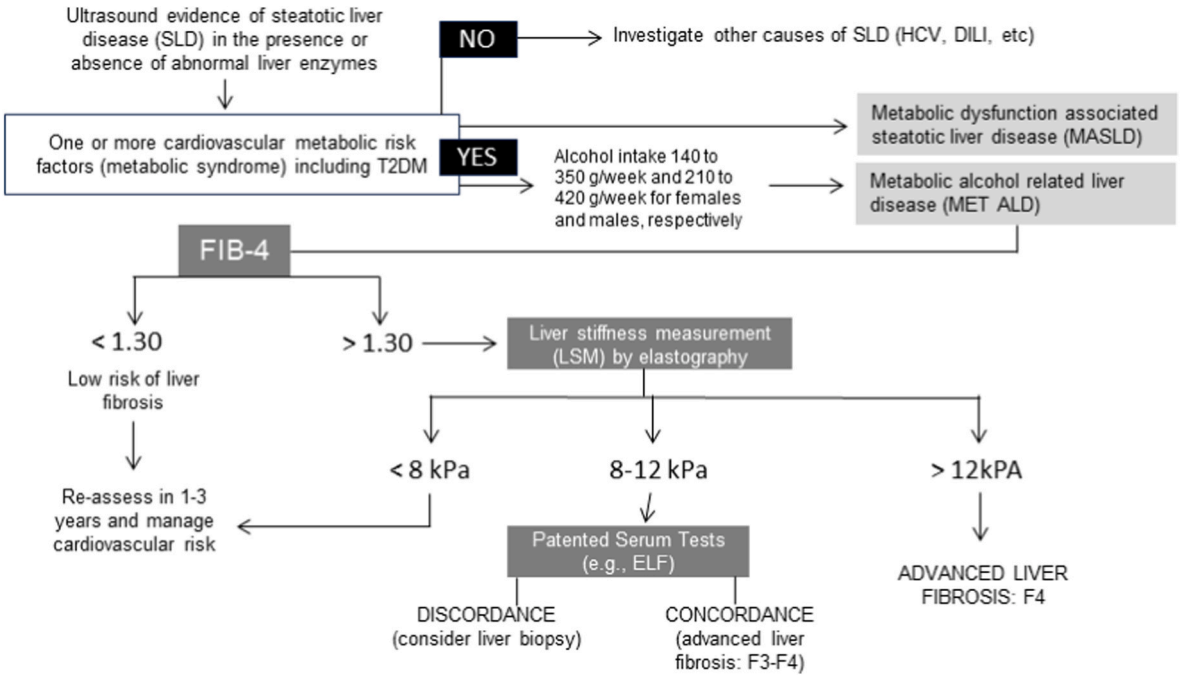

Fig. 4. Diagnostic flowchart for NAFLD. The terminology employed reflects the new nomenclature for NAFLD (Rinella et al., J Hepatol, 2023).

stiffeness increases and the waves propagate faster. Stiffness is measured in kilo Pascals (kPa) and increased levels of stiffness correlate with the stages of tissue fibrosis. There are several types of ultrasound elastography: transient elastography (TE, also know as vibration controlled transient elastography, VCTE), 2 dimensional shear wave elastography (2D-SWE), and point Shear Wave Elastography (pSWE). The three elastographic tecnhiques are characterized by similar diagnostic accuracy for the assessment of liver fibrosis (AUROC for fibrosis stage F3/F4: VCTE 0.88, 2D-SWE 0.92, pSWE 0.92). This accuracy is sufficiently close to that of the gold standard magnetic resonance elastography (MRE, AUROC 0.97), which is only reserved to clinical trials in reason of the much higher cost and logistic requirements (Singh, et al., 2015; Hu et al., 2017; Xiao et al., 2017; Lin et al., 2020). Nonetheless, VCTE is still the most recommended tool to diagnose NAFLD and fibrosis. As TE has a 94–100% negative predictive value, it can rule out the individuals with no fibrosis with high accuracy (Castera, et al., 2019). The reccommendation on VCTE is also based on the fact that, although based on ultrasound waves, its use does not require expertise in liver ultrasound by the operator. In practical terms and according to the guidelines of the EASL (EASL, 2021), the threshold of liver stiffness by VCTE suggesting the presence of significant liver fibrosis is $\geq$ 8.0 kPa (Fig. 4). In a recent real-world multicenter study including a large number of NAFLD patients with liver biopsy, the presence of advanced liver fibrosis (F3–F4) was excluded by a liver stiffness (LS) $<$ 8.0 kPa with a sensitivity of 91% and was confirmed with a LS $\geq$ 12.0 kPa with a specificity of 92% (Papatheodoridi et al., 2021).

Despite the increasing effectiveness of non-invasive methods to predict the presence of fibrosis in NAFLD, the gold standard assessment for NASH and fibrosis requires the histological evaluation of the liver by performing a liver biopsy. This allows a more accurate evaluation of key pathological features such as hepatocyte balooning and inflammatory infiltration in addition to a more precise definition of the fibrosis stage.

Liver inflammation is considered a key driver in NASH disease progression, with up to 30 % of cases developing advanced fibrosis and eventually cirrhosis. This is associated with liver disease-associated death because of cirrhosis complications and increasing incidence of HCC. Regardless, the prognostic value of inflammation in NASH in terms of morbidity or mortality remains controversial. An initial systematic review of trials with paired biopsy specimens indicated that histological inflammation in the first biopsy sample independently predicts the progression to advanced fibrosis and cirrhosis in NASH (Argo et al., 2009). However, as previously highlighted, retrospective longitudinal trials suggest liver fibrosis, not inflammation, as the major prognostic factor of liver-disease associated mortality (Hagstrom et al., 2017). Histological assessment includes evaluation of the NAFLD activity score (NAS), developed by the NASH Clinical Research Network in 2005 (Kleiner et al., 2005), and the fibrosis stage. The NAS is a numeric semi-quantitative score aimed at establishing three histological features: steatosis (scored 0–3), lobular inflammation (scored 0–3) and ballooning (scored 0–2); their sum reflects the disease grade (total score, 0–8). The score also assesses the severity of fibrosis (scored 0–4) and enables assessment of the disease stage. This score has been and still is of major utility to identify the appropriate individuals for enrollment and as efficacy endpoints in therapeutic intervention clinical trials. However, liver biopsy has two main limitations: it is invasive and with potentially severe complications in addition of being an imperfect diagnostic tool with limitations related to sub-optimal sampling and inter- and intra-observer variability. These latter limitations and the lack of guidelines on the biopsy-reading process has led to an insufficient standardization across clinical trials with often inconclusive results and major difficulties in making a comparative analysis of different trials. Therefore, a major consensus refinement with regulatory authorities is warranted and urgently needed (Harrison et al., 2023b).

7.3. Novel diagnostic perspectives

7.3.1. Serum biomarkers

Novel "omics" technologies have been actively employed for the identification of novel non-invasive biomarkers for NAFLD patient identification, NASH versus NAFLD patient discrimination and fibrosis staging.

Oxidative stress is one of the key mechanisms of lipotoxicity-induced hepatic damage leading to lipid oxidation products detectable in serum samples. Lipidomics studies performed using mass spectrometry have identified fatty acids-derived products associated with NASH. Arachidonic acid oxidation products, including 11-hydroxyeicosatetraenoic acid (11-HETE), and linoleic acid oxidation products such as hydroxyoctadecadienoic acid (HODE) and 9 and 13 oxo-octadecadienoic acids

(oxo-ODE), have been identified as possible biomarkers of NASH. Linoleic acid and 13-HODE ratios have been added to other clinical parameters (age, BMI, and AST) to construct the so-called oxNASH panel. This panel has a sensitivity of 81% and specificity of 97% in NASH versus not NASH discrimination (Engin, 2017).

Circulating extracellular vesicles (exosomes and ectosomes) containing miRNAs, mRNAs, proteins, and DNA molecules have been proposed as NAFLD biomarkers. Ectosomes are vesicles of various size (0.1–1 mm in diameter) that originate directly from the plasma membrane and are shed to the extracellular space. Ectosomes bind to recipient cells and deliver their informative cargo. An increase interaction between ectosomes and monocyte and natural-killer cell surfaces and a decrease in neutrophyl and endothelian cell surfaces have been observed in patients with NASH. In addition, production of exosomes and other extracellular vesicles are increased in this clinical setting, and it has recently been suggested that a specific protein signature in serum extracellular vesicles may be used as non-invasive diagnostic biomarkers (Povero et al., 2020).

Although the involvement of miRNAs has been highlighted in NAFLD pathogenesis very few studies have evaluated circulating miRNAs as biomarkers of fibrosis in NAFLD populations (Miyaaki et al., 2014; Sun et al., 2015). miR-122 was found to be upregulated in patients with fibrosis F2–F3 versus patients with fibrosis stages F0–F1, with an AUC of 0.61. Despite this not optimal AUC, miR-122 efficiency was higher than CK-18 (AUC: 0.49), ALT (AUC: 0.59) and AST (AUC: 0.64) in the prediction of the stage of fibrosis (Pirola et al., 2015).

7.3.2. Polygenic risk scores

NAFLD heritability is estimated between 20% and 70%, with substantial evidence suggesting the contribution of common genetic variants to the onset and progression of NAFLD (Sookoian and Pirola, 2017; Eslam et al., 2018; Romeo et al., 2020). Dongiovanni and co-workers reported on the development of a weighted polygenic risk score (polygenic risk score-hepatic fat content [PRS-HFC]), based on variants primarily increasing liver fat content, and showed that an increase in liver fat content is causally related to inflammation, ballooning, and fibrosis in patients with NAFLD (Dongiovanni et al., 2018). In addition, a genetic risk score combining 3 genetic variants (PNPLA3, TM6SF2, and HSD17B13) was associated with the risk of cirrhosis and HCC (Bianco et al., 2021; Gellert-Kristensen et al., 2020). More recently, the same group of investigators combined the PRS-HFC with fibrosis scoring systems in the UK Biobank and demonstrated a significant improvement in risk stratification and prediction of severe liver disease, especially in subjects at high-risk for NAFLD (De Vincentis et al., 2022). While more studies in the general population and in individuals at risk are required, these recent acquisitions open new perspectives in the development of powerful tools for predicting the risk of severe disease in patients with NAFLD and improving the clinical management and the stratification for clinical therapeutic trials.

8. Current/emerging therapies

When considering the treatment of NAFLD it is important to prioritize the assessment of metabolic risk factors in patients with incidental findings of fatty liver independently of liver enzyme abmormalities and liver-related symptoms. Indeed, it is evident that patients with NAFLD have increased cardiovascular morbidity and mortality (Targher et al., 2016). Moreover, advanced liver fibrosis is associated with increasing number of metabolic comorbidities (Wong et al., 2019). Accordingly, early identification and treatment of individual components of the metabolic syndrome are critical in preventing both cardiovascular and liver-related mortality.

From the point of view of Hepatology specialist care, once a diagnosis of NASH has been achieved treatment should be directed at improving clinical outcomes, i.e., decrease NASH-related mortality, reduce progression to cirrhosis and HCC occurrence. The resolution of the histological lesions defining NASH and especially reduction of liver fibrosis are now widely accepted as a surrogate endpoint, particularly in clinical trials. On the other hand, robust data correlating NASH resolution with hard clinical outcomes have been lacking, but NASH resolution has been accepted as steatohepatitis is the disease driver that leads to fibrosis. In addition, recent data have shown that NASH resolution correlates with fibrosis improvement (Brunt et al., 2019).

8.1. Lifestyle management of metabolic risk factors

Given the common association of metabolic syndrome and NAFLD with excessive body weight (BMI >25 kg/m^2) and frank obesity (BMI >30 kg/m^2), the first therapeutic effort is directed at the reduction of body weight. The only treatment which has robustly been proved to ameliorate liver damage in patients with NAFLD without severe liver fibrosis is represented by weight loss achieved through diet. EASL guidelines recommend diets that have a 500–1000 kcal/d deficit, exclusion of NAFLD-promoting components and macronutrient composition adjusted according to the Mediterranean diet. Diet should be combined with physical activity (150–200 min/wk of moderate intensity aerobic exercise or resistance training in 3–5 sessions). In overweight or obese patients, the goal is to achieve a 7%–10% weight reduction, which results in improvement of liver enzymes and histology (EASL, EASD, EASO., 2016). Recently, intermittent fasting diets have gained popularity among healthy individuals and have been proposed as a safe and effective treatment for the metabolic syndrome in experimental and in a few human studies (Pugliese et al., 2022; Memel et al., 2022).

8.2. Pharmacological intervention

Several drugs for limiting NAFLD development and progression have been examined, although none is currently specifically licensed for NAFLD treatment. However, the knowledge on the cascade of mechanisms leading to metabolic injury and the activation of inflammatory and fibrogenic pathways has greatly expanded over the past decade and the introduction of pharmacological approaches targeting several cellular components or molecular pathways of NASH has become increasingly realistic (Tacke et al., 2023). Indeed, the positive results from FXR agonist obeticholic acid or the thyromimetic resmetirom registrational trials have been recently released (Younossi et al., 2019; Madrigal Announces, 2023). Moreover, combinations of agents targeting different pathways have been proposed and early results of these studies are becoming available. Another aspect contributing to a more effective approach has been the agreement between the two key regulatory agencies, the FDA and EMA, that a substantial histological improvement, defined as resolution of NASH and/or improvement in the stage of fibrosis, will be sufficient to yield drug approval and long-term benefit (i.e., overall reduction of liver- or cardiovascular-related events, mortality, or liver transplantation) (Loomba R et al., 2022). Overall, this decision has practically removed hepatocellular ballooning, the most debated histological feature of NAFLD, as a convincing target perspective. Indeed, although ballooning seems to be a histological feature of progressive disease, its detection and quantification is extremely subjective and therefore not suitable as an effective treatment endpoint (Brunt et al., 2022).

8.2.1 Targeting hepatic metabolic injury

The first group of pharmacological agents to be considered are those directed at targeting hepatocellular metabolic injury and stress leading to the activation of inflammatory cascades. Many of these agents originate from the treatment metabolic conditions such as T2D or dyslipidemia or obesity and are now in trials for NASH. Among the compounds targeting key metabolic pathways in the liver and are in late stage development, is important to mention those affecting lipogenesis (e.g., aramchol, inhibitors of acetyl-CoA carboxylase or fatty acid synthase),

energy availability (e.g., glucagon-like peptide 1 [GLP-1] receptor and/or glucagon agonists) or lipid handling (e.g., fatty acid β-oxidation via nuclear receptors, such as THR mimetics like resmetirom (Chew et al., 2022). In addition, the beneficial metabolic effects of long-chain omega-3 fatty acids can be maximized by the administration of structurally engineered fatty acids, such as icosabutate, which are characterized by improved resistance to peroxidative processes, when compared to long-chain omega-3 fatty acids (Fraser et al., 2022).

The next step is to target the metabolic dysfunction in the hepatic cellular microenvironment including non-parenchymal cells in addition to hepatocytes. Hepatic cells are metabolically regulated by a variety of nuclear receptors (ligand-controlled transcription factors) activated by a variety of ligands including hormones, lipids and bile acids and regulating glucose, fat, and cholesterol homeostasis (Fuchs and Trauner, 2022). The most extensively exploited for the treatment of NASH are peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), FXR and THR (particularly THR-b) (Wirth et al., 2022; Francque S et al., 2021a). Among PPAR agonists, Lanifibranor, one of the most recently developed potential anti-NASH drugs, is the first PPAR-pan agonist, targeting all three PPAR isotypes. Lanifibranor was found to reduce liver steatosis, inflammation, and hepatocyte ballooning in different mouse models of NASH. In addition, a decrease in the fibrotic response was also observed in the MCD diet-fed mouse model and CCl_4-induced fibrosis in mice (Wettstein et al., 2017). These positive effects were also shown in a phase 2b, double-blind, randomized, placebo-controlled trial, patients with noncirrhotic, highly active NASH (Francque et al., 2021b).

Since the metabolic syndrome is the systemic pathophysiological background of NAFLD and NASH, extrahepatic mediators, e.g., from the gut, adipose tissue, or the endocrine system, are also being evaluated as drug targets for the treatment of NASH. Along these lines, interfering with gut-derived signals could be proposed as a method to reduce inflammatory responses in the liver, since the gut and liver form an anatomically and functionally connected unit, the so-called "gut-liver axis". Accordingly, reversing gut dysbiosis in NAFLD using pre-/pro-/antibiotics or faecal microbiota transplantation would reduce these inflammatory signals and improve the integrity of the gut barrier function. This possibility is supported by a large amount of preclinical data in animal models but substantial evidence from trials in patients with NASH is lacking (Albillos et al., 2020). On the other hand, the gut can also produce "anti-inflammatory" mediators with beneficial effects to the liver, including hormones such as GLP-1 and fibroblast growth factor (FGF)-19. These hormones act mainly indirectly on the liver by reducing energy supply and/or improving hepatocyte metabolism and are already in clinical use for metabolic diseases (e.g. GLP-1 receptor agonists, such as semaglutide or liraglutide, GLP-1/GIP [glucose-dependent insulinotropic polypeptide] dual agonists, such as tirzepatide) or in advanced clinical development (e.g, GLP-1/glucagon dual agonists, such as cotadutide, or the FGF-19 mimetic aldafermin) (Boland et al., 2020).

8.2.2 Targeting the inflammatory response in NASH

Targeting the inflammatory response in NASH is not a straightforward process since there is still limited understanding of the pathophysiology of inflammation in NAFLD. Indeed, an oversimplified definition of inflammation is often applied that does not account for the distinct functions of immune cells allowing to differentiate between disease-promoting and resolving mechanisms of inflammation. In addition, inflammation in NAFLD is highly dynamic and tends to fluctuate between peaks of activity and resolution. However, proceeding with a logical approach, the current pharmacological strategies targeting the initiation and maintenance of inflammation in NAFLD/NASH can be summarized in two main attempts: a) inhibition of the primary recognition of injury and subsequent activation of inflammatory cascades, and b) target the recruitment of inflammatory immune cells and/or modulate the complex immune cell crosstalk.

Active ongoing research is addressing the possibility of interfering with TLRs, the inflammasome and the relative inflammatory signalling. In this context, main cellular targets are liver non-parenchymal cells (sinusoidal endothelial cells, HSCs) and liver-resident immune cells (Kupffer cells and innate lymphocyte populations). In addition, since the NLR family pyrin domain containing 3 (NLRP3) inflammasome is a central driver of inflammation and an inducer of inflammatory ("pyroptotic") cell death in NASH, pharmacological inhibitors of NLRP3 activation have been proposed but are still in early-stage development (Mangan et al., 2018).

Another approach is targeting immune cell recruitment by blocking chemokines released by damaged hepatocytes and activated non-parenchymal cells and interfering with chemokine receptor function. Within this context, the chemokine CCL2 and its receptor CCR2 have been tested as therapeutic targets in NASH by employing the dual CCR2/CCR5 inhibitor cenicriviroc (Wiering and Tacke, 2022). However, despite initial promising results cenicriviroc did not demonstrate sustained antifibrotic efficacy after one year of treatment in a larger phase III clinical trial leading to the termination of its development as a monotherapy in NASH. Alternative anti-chemokine strategies, i.e., the CCR2 inhibitor propagermanium or an RNA-aptamer molecule inhibiting CCL2 (NOX-E36) (Tacke, 2017) have not been tested in patients with NASH. In any case, the example of cenicriviroc highlights the unlikeliness to achieve a successful strategy targeting a single inflammatory pathway in NASH in reason of the redundancy of the chemokine system, with many ligands and receptors that have overlapping targets, combined with the overlying functions and adaptability to environmental signals of immune cell populations.

A further proposed strategy is the modulation of the immune cell crosstalk by targeting platelets, neutrophils, and lymphocytes. However, this area of intervention is still very premature and with very slow development. Observational studies have shown some benefits of antiplatelet agents in patients with NASH in generally treated for cardiovascular indications, while pharmacologic inhibition of the hyaluronan-CD44 anchoring of platelets has been shown to be an effective anti-inflammatory strategy to attenuate NAFLD in preclinical models (Malehmir et al., 2019).

8.2.3 Targeting fibrogenesis in NASH

As previously introduced, the severity of hepatic fibrosis in NASH is the main predictor of liver-related, as well as overall, morbidity and mortality. It is therefore understandable that the development of antifibrotic agents represents a high priority. The development of agents able to directly affect the fibrogenic action of the main cellular effectors (e.g., HSCs) has a long history but not yet consistent results in reason of the difficulty of therapeutically targeting these cells.

Within this not very exicting landscape, promising results have been obtained blocking the activation of TGF-β by targeting alphaV-containing intergrins in preclinical models of NASH fibrosis (Henderson et al., 2013). On the other hand, several integrin inhibitors are currently in clinical trials (mostly for pulmonary fibrosis), including PLN-74809, a dual avb6/avb1 integrin inhibitor, in patients with liver fibrosis (Rahman et al., 2022).

On a different aspect, there is increasing evidence that some of the pharmacological agents addressing metabolic disfunction in the metabolic syndrome and currently in phase II/III clinical development may have anti-fibrotic properties. Among those targeting *de novo* lipogenesis, acetyl-CoA carboxylase and fatty acid synthase inhibitors, have been shown to suppress fibrogenesis directly. Along these lines, the acetyl-CoA carboxylase inhibitors firsocostat and clesacostat (PF-05221304) were able to reduce HSC activation in cell culture and liver fibrosis in animal models, independently of their anti-steatotic effects (Bates et al., 2020; Ross et al., 2020). Very similar observations were obtained in in vitro cultures of HSC with aramchol, an inhibitor of the lipogenic enzyme stearoyl-CoA desaturase 1 (Bhattacharya et al., 2021). Finally, hexokinase inhibitors (e.g., PF-06835919) or SGLT2 (sodium glucose linked transporter 2) inhibitors like empaglifozin, licoglifozin or

dapaglifozin may attenuate HSC activation and fibrogenesis. However, clinical evidence on SGLT2 inhibitors as a treatment specifically for NASH is currently limited (Harrison et al., 2022).

8.2.4. Towards combination therapies and personalized approach

In reason of the systemic pathophysiological background of NAFLD it is conceivable that the treatment of NASH will proceed towards a combinatorial strategy by combining metabolically acting drugs with agent able to reduce the inflammatory and fibrogenic process. This approach needs significant additional work since clinical trials using combinations have been disappointing despite promising preclinical data in mouse models.

In any case, possible examples of applications include weight-loss medications such as glucagon-like peptide 1 (GLP1) receptor agonists and SGLT2 inhibitors with additional benefits in management of diabetes and cardiovascular disease prevention. It is auspicable that the new nomenclature of NAFLD will also provide more clarity in the stratification of patients to be recruited in clinical trials. Overall, the abundance of pharmacological options at the orizon will possibly allow a personalized approach in the future.

9. Conclusions

The knowledge on the pathophysiology of NAFLD has considerably expanded over the past decade highlighting a high level of complexity. On one side is the development of liver steatosis in the context of a systemic metabolic derangement and its impact on the clinical manifestations and outcomes of the metabolic syndrome. On the other is the development of a fibrogenic chronic liver disease with possible evolution to liver cirrhosis. Several mechanisms responsible for the progression from “simple fatty liver” to extensive chronic hepatocellular damage, inflammation and fibrosis have been elucidated and currently represent targets for drug development. Research in this context has also evidenced the importance of a genetic mutations predisposing to chronic liver disease not only in NAFLD but also in other conditions of chronic hepatocellular and biliary damage. Identifying the presence of NAFLD and its possible evolution in a fibrogenic chronic liver disease represents a major public health issue requiring increasing sensibilization in general practice physicians and a multidisciplinary approach in secondary and tertiary care.

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